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On the Cover

Ellis Wilson (1899–1977)
Caribbean Bird Vendor (1953)
Oil on canvas (91.44 cm × 60.96 cm)
Hampton University Museum,
Hampton, Virginia, USA

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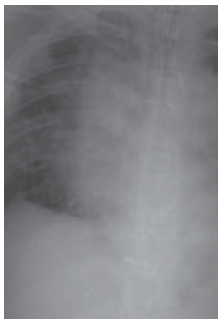

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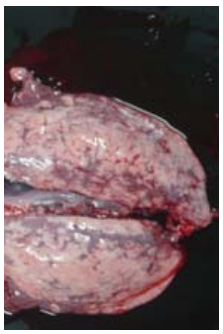

Effects of Coronavirus Infections in Children

Nicola Principi, Samantha Bosis, and Susanna Esposito

The isolation of the coronavirus (CoV) identified as the cause of severe acute respiratory syndrome and the detection of 2 new human CoVs (HCoV-NL63 and HCoV-HKU1) have led to studies of the epidemiology and clinical and socioeconomic effects of infections caused by all HCoVs, including those known since the late 1960s (HCoV-229E and HCoV-OC43). HCoV infections can be associated with respiratory and extrarespiratory manifestations, including central nervous system involvement. Furthermore, unlike other RNA viruses, HCoVs can easily mutate and recombine when different strains infect the same cells and give rise to a novel virus with unpredictable host ranges and pathogenicity. Thus, circulating HCoVs should be closely monitored to detect the spread of particularly virulent strains in the community at an early stage and to facilitate the development of adequate preventive and therapeutic measures.

Human coronaviruses (HCoVs) have been known since the late 1960s as a group of viruses capable of infecting humans and animals (*1*). In a wide variety of animals, they cause respiratory, enteric, hepatic, and neurologic diseases that, in some cases (especially when they infect the young), can be severe (*1*). However, until the pathogen identified as the cause of severe acute respiratory syndrome (SARS) was isolated (*2*), the previously known HCoVs (HCoV-229E and HCoV-OC43) were considered to play a marginal clinical role in pediatrics. This conclusion was made mainly because, on the basis of the data available at the time, HCoVs were believed to cause only mild upper respiratory tract infections (URTIs) in children and that only in premature infants and children with a chronic underlying disease could severe lower respiratory tract infections (LRTIs) develop (*3*). Moreover, no importance was placed on reports that suggested a possible relationship with the development of extrarespiratory problems, including central nervous system (CNS) involvement, in which HCoVs can persist and play a role in causing chronic neurologic disorders (*4*). Consequently, the circulation of HCoVs was not monitored, and no attempt was made to develop vaccines or drugs that were active against the viruses.

The identification of SARS-CoV and the isolation of 2 novel HCoVs in humans (HCoV-NL63 and HCoV-HKU1) (*5*,*6*) have led to several studies of the epidemiology and clinical and socioeconomic effects of HCoV infections, which were greatly facilitated by the availability of modern molecular biology methods that enable direct viral identification in respiratory secretions (*7*–*26*). Interest was strengthened by the demonstration that SARS could be considered a zoonotic infection because, after it was described and the causative agent identified from patients in the People's Republic of China, SARS-CoV–like viruses were isolated from caged animals, including palm civets and raccoon dogs in wildlife markets of the same Chinese province (*27*). This finding and the subsequent independent discovery of SARS-CoV–like viruses in horseshoe bats indicated that wild animals could be the reservoir of these viruses and that, in a suitable environment, they could infect humans and cause epidemics. New data concerning old and the new HCoVs raise the question of whether HCoVs may be more clinically important in children than was previously thought, thus indicating the need for a systematic evaluation of their circulation and the availability of preventive and therapeutic measures.

Epidemiology of HCoV Infections in Children

A profound difference exists between the epidemiology of the infections caused by SARS-CoV and that of all other HCoV infections. SARS-CoV emerged in November 2002 and disappeared in April 2004 (*28*). During these 18 months, it was isolated in many countries, some of which

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were very distant from each other. However, the total of 8,098 cases of SARS diagnosed worldwide (*28*) is substantially fewer than the number usually found during epidemics of the most common respiratory viruses, such as respiratory syncytial virus (RSV) and influenza viruses (*18*).

Furthermore, seroepidemiologic studies of high-risk and low-risk residential areas have clearly shown that the prevalence of immunoglobulin G against SARS-CoV was low in children and adults (*29*); this result indicates that SARS-CoV not only had a restricted period of circulation but also that it had limited spread. Proportionally fewer children were involved: <5% of all cases were diagnosed in patients <18 years of age (*28*). The biology of SARS and its low level of transmissibility seem to be the main reasons for the low risk for contagion in children. In most of the areas in which outbreaks occurred, healthcare workers and adult patients were mainly involved and, because they were immediately hospitalized, the risk for the infection spreading to children was greatly reduced because they are not usually allowed to visit hospitals (*28*). This hypothesis seems to be further supported by the fact that the early detection and isolation of symptomatic patients were the most important measures in controlling the SARS epidemic.

Unlike SARS-CoV, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 have been in continuous circulation since their first isolation and every year cause a large number of infections that more frequently involve children than adults (*3*,*5*–*26*). In particular, the data regarding the earlier-appearing HCoVs indicate that they are distributed throughout the world and mainly circulate during the winter and early spring, with outbreaks occurring every 2–4 years (*3*). Whenever HCoVs have been sought in studies of respiratory infections in infants and children, they have been found, although generally less frequently than other respiratory viruses such as RSV and influenza (*18*,*30*). However, the real importance of HCoV-229E and HCoV-OC43 in clinical practice has not been fully defined because the collected data are often discordant. In 1974, McIntosh et al. found that the global incidence of LRTIs due to HCoV-229 and HCoV-OC43 in hospitalized children was no higher than 3.8% (*31*). Later studies have shown that when all respiratory infections are considered, the etiologic prevalence of these pathogens in pediatric patients can be significantly higher, varying from ≈5% to >30% (*7*,*15*).

An overall evaluation of the available data suggests that these differences can be attributed to differences in research methods. The infections caused by these viruses are more common from November through March; more frequently affect children <5 years of age, those examined in the community, and those without underlying risk factors; and are more often identified with serologic methods (*32*,*33*). In this regard, 2 recent surveys that used serologic methods alone found previous HCoV-229E infection in 42.9%–50.0% of children 6–12 months of age (*32*) and in 65% of those 2.5–3.5 years of age (*33*). Similarly, in 75% of the cases, children 2.5–3.5 years of age had antibodies against HCoV-NL63 (*33*). All these factors can explain why the lowest incidences of HCoV-229E and HCoV-OC43 infections are usually found when the study population includes older children or adolescents, patients with underlying severe chronic diseases or hospitalized patients, when only highly symptomatic infections are considered, and when the study is conducted during the whole year.

Moreover, what has been clearly shown is that the original HCoVs are commonly detected in childhood and frequently isolated in the nasopharyngeal secretions of children with respiratory infection. In some cases, co-infections with other respiratory viruses, mainly RSV, influenza viruses, and human metapneumovirus, have also been found (*4*,*7*–*23*). However, the real incidence of HCoV-229E and HCoV-OC43 co-infections with other respiratory pathogens has not yet been defined because only a few of the published studies were planned to identify all the main respiratory viruses.

Similar conclusions can be drawn in relation to the more recently identified HCoVs. HCoV-NL63, which can be found in 1.0%–9.3% of nasopharyngeal aspirates from patients with RTIs (*7*–*23*), circulates throughout the world (predominantly during the winter in temperate regions), infects mainly younger children and subjects with underlying severe chronic diseases, and is more frequently found in nonhospitalized children (online Appendix Table, www.cdc.gov/EID/content/16/2/183-appT.htm). Although it was not isolated until 2000, HCoV-NL63 has probably been circulating for some time because one of the first detections occurred in a sample of nasopharyngeal secretions collected from a child with pneumonia that had been kept in a freezer since January 2003 before evaluation (*5*).

HCoV-HKU1 was identified in 2005 and has once again been found in nasopharyngeal secretions of children and adults with respiratory infections in countries that are very distant from each other. Its incidence varies from <1% to 6% (Table) (*6*,*20*,*24*–*26*), and seroepidemiologic surveys based on antibodies reacting with the recombinant HKU1 nucleocapsid protein suggest that infection may be relatively common in humans, although generally asymptomatic (*32*,*33*). HCoV-NL63 and HCoV-HKU1 are often associated with co-infections with other respiratory viruses, mainly RSV and influenza viruses (*7*–*26*).

Clinical Manifestations of HCoV Infections in Children

Respiratory Problems

It is well known that all HCoVs cause respiratory infections. SARS-CoV is the most aggressive, although the

Table. Main studies of the epidemiology and clinical relevance of HCoV-HKU1 in infants and children*

Study	Location and period	Population	No. samples tested	No. (%) patients with positive test results	Comments
Lau et al. (*20*)	Hong Kong; 2004 Apr–2005 Mar	629 children with RTIs, 6 mo–5 y; inpatients	629	10 (1.6)	11 patients with URTIs, 1 with pneumonia, 1 with bronchiolitis, 5 with febrile seizures; 3 with underlying disease
Vabret et al. (*24*)	Canada; 2005 Feb–Mar	83 children with RTIs, <5 y; negative for RSV, influenza A/B, PIV 1–3, adenovirus; inpatients	83	5 (6.0)	3 patients with gastroenteritis, 1 with febrile seizures; mean age 26 mo
Sloots et al. (*25*)	Australia; 2004 May–Aug	259 children with RTIs, <5 y; inpatients and outpatients	259	10 (3.8)	1 patient with co-infection
Talbot et al. (*34*)	USA; 2001 Oct–2003 Sep	1,055 children with RTIs, <5 y; inpatients	1,055	4 (0.4)	Mild episodes

*HCoV, human coronavirus; RTI, respiratory tract infection; URTI, upper respiratory tract infection; RSV, respiratory syncytial virus; PIV, parainfluenza virus.

disease seems to be substantially less severe in children than in adults. In patients <12 years of age, the clinical course of SARS was generally milder and shorter than in those ≥12 years: no death was reported, only 5% of the infected children were admitted to an intensive care unit, and <1% required mechanical ventilation (*28*). Leung and Chiu found that several children with SARS-CoV infection recovered without any sequelae after receiving supportive therapy alone (*36*). The only pediatric patients with severe respiratory problems associated with SARS-CoV infection were >12 years (*36*). The clinical picture in persons <12 years was similar to that caused by other respiratory viruses, including influenza viruses. Moreover, the extrarespiratory manifestations of SARS-CoV infection described in adults (hepatitis and CNS dysfunction) have never been reported in children.

The clinical role of all non-SARS CoVs seems to be similar: in most healthy children: they cause URTIs that spontaneously disappear in a few days. This finding is clearly shown by our data indicating no difference in the incidence and clinical severity of the diseases associated with HCoV-229E, HCoV-OC43, and HCoV-NL63. Regardless of the HCoV causing the infection, ≥50% of the children had a common cold or pharyngitis, and laboratory and radiologic investigations were required in <15% (*18*). Moreover, we also found that the socioeconomic effects of these viruses on the families of the infected children was marginal: the viruses spread significantly less than influenza viruses among household members, caused only a limited number of similar infections in the family, and led to fewer lost working or school days (*18*).

Although possible, the association of non-SARS HCoV infection and LRTI is uncommon in healthy children. In most published studies, the incidence of pneumonia or bronchiolitis was <5% (*7–26*,*34*). Differences in the incidence of LRTIs among studies can at least partially be attributed to the different prevalence of co-infections. Gerna et al. found a high incidence of HCoV-229E, HCoV-OC43, and HCoV-NL63 infections in infants and children with bronchitis, bronchiolitis, or pneumonia, but most of the LRTIs were demonstrated in children co-infected with HCoVs and other respiratory viruses (*37*). Furthermore, the method used to collect respiratory samples can also play a role in explaining the greater incidence of LRTIs (*11*,*14*,*16*,*22*). In this regard, it is important to emphasize that when only hospitalized patients (with, consequently, only the most severe cases) are enrolled, the incidence of LRTIs seems greater (*34*,*37*).

Unlike in healthy children, the development of severe clinical features after infection with non-SARS HCoV is relatively common among newborns, premature and low birthweight infants, and children at risk because of underlying health problems. Gagneur et al. described 3 HCoV-229E–related outbreaks in a pediatric and neonatal intensive care unit in France during 1998 (*38*), and 75% of the neonates and 92% of the extremely premature infants were symptomatic. Kuypers et al. studied the contribution of non-SARS HCoVs to acute RTIs and found that several children with isolated HCoV disease had an underlying medical condition (*21*).

Recently collected data concerning the individual viruses indicate that HCoV-NL63 may be more frequently associated with croup than with HCoV-229E or HCoV-OC43. Van der Hoek et al. found that 9 (45%) of 20 children infected by HCoV-NL63 alone, and 12 (25%) of 49 HCoV-NL63–positive children as a whole, had croup, compared with 54 (6%) of 900 HCoV-NL63–negative children ($p<0.001$) (*16*). Wu et al. (*22*) and Han et al. (*35*) also reported a high prevalence of croup in children infected with HCoV-NL63. Furthermore, other reports indicate that both HCoV-NL63 (*9*,*12*,*15*,*16*,*21*) and HCoV-HKU1 (*20*,*26*) are associated with the development of bronchiolitis and wheezing.

Extrarespiratory Problems

As mentioned above, SARS-CoV does not seem to cause extrarespiratory problems in children, but all of the other HCoVs can be associated with signs and symptoms involving organs and systems other than the respiratory tract. Abdominal pain, emesis, and diarrhea can be the first signs and symptoms of an acute infection due to non-SARS CoVs. These manifestations have been reported, particularly in the cases of HCoV-OC43 and HCoV-NL63, and seem to be the direct consequences of viral invasion of the intestinal mucosa, as suggested by the presence of HCoV-like particles in the stool samples of many patients with acute disease (*3*,*7*–*23*).

Non-SARS CoV infections have also been associated with acute and chronic CNS diseases (*4*,*20*), although no clear evidence has shown that the viruses played a direct causative role. Nevertheless, some evidence exists of a possible relationship between HCoV infection and CNS damage. HCoV-229E and HCoV-OC43 infections have been associated with the development of various chronic neurologic disorders, including multiple sclerosis, because these viruses have been found more frequently in the autopsied brain tissue of patients with these diseases more frequently than in healthy patients (*4*). A possibly causative role of HCoV-OC43 in determining chronic brain damage is further supported by the fact that chronic demyelinisation of mouse CNS can be induced by infection with another CoV, mouse hepatitis virus (MHV), which belongs to the same antigenic group as HCoV-OC43 and has structural similarities with it (*39*).

Because MHV induces the secretion of pro-inflammatory molecules, such as interleukin-1β (IL-1β), tumor necrosis factor, IL-6, and macrophage-inflammatory 1β, during the infection of neural cells, HCoV-OC43 may act similarly in the CNS of infected children and lead to severe brain damage. Furthermore, SARS-CoV (which has many genetic similarities to both viruses) seems to cause lung damage by activating the same pro-inflammatory molecules, because a particularly high level of circulating IL-1β has been found in children with SARS (*34*).

An association of acute neural disease with HCoV infection has been clearly demonstrated by the detection of HCoV-OC43 in the cerebrospinal fluid of a child presumed to have acute disseminated encephalomyelitis, and the frequent association between HCoV-HKU1 infection and the development of febrile seizures seems to lead to the same conclusion. Lau et al. studied 10 children infected by this virus and found that half were affected by febrile seizures, the highest prevalence among all the HCoVs (*20*). Because the fever in all of these children was not particularly high and lasted for a shorter period than fever associated with other viral respiratory infections, it was considered unlikely that all were simple febrile seizures, but possible that they may represent specific neurologic damage induced by HCoV-HKU1 or that the virus may trigger a negative immune response.

Finally, Esper et al. identified HCoV-NL63 in respiratory specimens from 8 (72.7%) of 11 children with Kawasaki disease (KD) and in only 1 (4.5%) of 22 age-matched controls, thus suggesting that KD may be triggered by a response to HCoV-NL63 infection (*40*). However, the findings of other studies do not support this observation, and so the question of the causative role of HCoV-NL63 in the development of KD remains unanswered.

Assessment of the Importance of Known HCoVs in Children

Superficial analysis of all of the available data concerning the effects of HCoVs in children suggests that the assessment of the importance of HCoVs made before SARS-CoV was identified can still be considered valid. In general, all of these viruses (including SARS-CoV) have been confirmed as mainly respiratory viruses with limited clinical relevance in children. They cause mainly URTIs, are not frequently isolated in hospitalized children (*7*–*26*), and, because they are rarely transmitted to other household members, have a marginal socioeconomic effects on families (*18*). Even SARS-CoV infection, which had a dramatic effect on adults, was mainly associated with relatively mild disease in almost all patients <12 years of age (*28*,*34*). Moreover, most children with a diagnosis of severe respiratory syndrome in whom a HCoV was isolated were coinfected by other respiratory viruses (*7*–*26*). These finding suggest that the severity of the respiratory disease at least in some of these cases was attributable to the second virus.

Because only premature infants, neonates with a low birthweight, and children with an underlying severe chronic disease are at risk of experiencing a severe respiratory problem associated with HCoV infection (*21*,*37*), we could conclude that no further studies of the role of HCoVs in children are needed because what is already known is enough to make such investigations superfluous. Furthermore, on the basis of the data regarding the natural outcome of respiratory infections, developing vaccines or specific drugs appear to be unnecessary.

However, different conclusions can be drawn when the global spectrum of the diseases caused by these viruses in animals and humans is considered. It is now well known that an enormous reservoir of CoVs exists among animals, particularly horseshoe bats, and that CoV isolates recovered from animals in China have up to 99.8% nucleotide identity with SARS-CoV (*27*). Because CoVs can easily mutate, this means that (as in 2003) sustained exposure to the infected animals can lead to a SARS-like CoV strain that is newly adapted to infect humans and capable of causing the reappearance of SARS. Moreover, it has been shown

experimentally and in nature that all CoVs undergo a high rate of genetic mutations and can recombine when 2 different strains infect the same cells (*1*). This finding means that it is theoretically possible that future situations similar to those involving SARS-CoV may involve CoVs that currently infect only some animals, thus leading to novel viruses with unpredictable host ranges and pathogenicity.

Phylogenetic analyses of the genes spanning the HCoV-HUK1 genome suggest that this virus may be the result of a recombination event between related but distinct HCoVs (*6*) and that SARS-CoV may have originated from a unique recombination (*2*). In this regard, the behavior of CoVs could be quite similar to that of influenza viruses, for which genetic changes and recombinations of avian or swine strains are required to allow them to cross the species barrier and replicate in humans to cause a pandemic.

Consequently, as is usually the case with influenza, a systematic evaluation of the characteristics of CoVs should be planned. Patients with severe respiratory syndrome seem to be the best target for this kind of evaluation and, in this population, studies of children (in whom the incidence of infection is higher) may also have application to adults because the findings may lead to a reduction in the risk for the spread of particularly virulent HCoV strains.

In addition to the risk for a pandemic related to the reappearance of SARS or other new CoVs, the data regarding the possible relationship between HCoV infection and CNS diseases also suggest the need for a systematic evaluation of the circulation of CoVs. If ≥1 HCoVs are demonstrated to play a real role in causing some of the CNS diseases with which they have been associated, substantial changes would be required in our diagnostic, prophylactic, and therapeutic approaches to many neurologic illnesses in children.

Conclusions

HCoV infections can be associated with respiratory and extrarespiratory manifestations, including central nervous system involvement. The clinical and genetic characteristics of circulating HCoVs in the pediatric population should be monitored to detect the spread of particularly virulent HCoV strains in the community at an early stage and, if required, to facilitate the development of adequate preventive and therapeutic measures.

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Dr Principi is professor of pediatrics at the University of Milan. His research activities have been primarily dedicated to the study of pediatric pharmacology and pediatric infectious diseases, particularly respiratory tract infections, vaccines, and HIV infection.

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Imported Methicillin-Resistant *Staphylococcus aureus*, Sweden

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Countries such as Sweden that have a low prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) offer the opportunity to discern and study transmission of imported cases of MRSA. We analyzed 444 imported cases of MRSA acquisition reported in Sweden during 2000–2003. Risk for MRSA in returning travelers ranged from 0.1 (95% confidence interval [CI] 0.01–0.4) per 1 million travelers to Nordic countries to 59.4 (95% CI 44.5–79.3) per 1 million travelers to North Africa and the Middle East. Most imported cases (246, 55%) were healthcare acquired, but regions with the highest risk for MRSA in travelers showed a correlation with community acquisition (r = 0.81, p = 0.001). Characteristic differences in MRSA strains acquired were dependent on the region from which they originated and whether they were community or healthcare acquired. Knowledge of differences in transmission of MRSA may improve control measures against imported cases.

Global transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) has been the subject of many studies. Conclusions have commonly been drawn from the occurrence of identical or similar strains in different countries or regions (*1*–*6*). Studies of individual MRSA cases linked to international travel have often been in the form of case reports, case series, or other descriptive reports (*7*–*11*). Only a few small analytical or population-based studies have been published (*12*–*14*). In countries with a high prevalence of MRSA, imported cases of MRSA are not easily distinguished from the domestic background prevalence. Thus, low prevalence countries, such as Sweden, offer a better opportunity to discern and study imported cases of MRSA.

The primary purpose of our study was to use validated data from the surveillance system in Sweden to analyze the risk for MRSA among Swedish residents traveling abroad, internationally adopted children, and immigrants. A secondary purpose was to determine whether different types of MRSA were acquired in different countries or regions.

Materials and Methods

Data on MRSA Cases in the Statutory Swedish Communicable Disease Notification System

The study was reviewed and approved by the Regional Ethics Approval Board in Stockholm (Protocol 2005/4:10). MRSA cases (infections as well as carriage) are notified in parallel to the Swedish Institute for Infectious Disease Control (SMI) by the doctor caring for the patient and by the bacteriological laboratory having isolated the MRSA strain, using a unique personal identification number issued to all Swedish residents and used in all contacts with the health care system. At SMI, a case record is created after the arrival of the first notification. Any subsequent notifications are merged with the initial case record. After completion of the epidemiologic investigation and contact tracing around each MRSA case, the epidemiologic notification form data are validated by SMI with input from MRSA contact persons, who are public health representatives involved in control efforts locally

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in the respective counties (*15*). The validated variables used in the study were age, sex, transmission setting (healthcare acquired [HA] or community acquired [CA]), country of acquisition, and purpose of travel (leisure or work related) (*16*). If a person infected with MRSA had been abroad ≤6 months before MRSA was diagnosed and either had a strain that was present in other persons from that region or had one that was previously unknown in Sweden and no likely source of MRSA in Sweden was found in the epidemiologic investigation, that person was considered to have acquired MRSA abroad. If there was a reasonable possibility that MRSA could have been acquired either domestically or abroad, country of acquisition was considered unknown. If the person infected with MRSA had been admitted to or had worked in a healthcare setting during his or her stay abroad, he or she was considered to have a case of HA MRSA. Information in the study database on whether a person had MRSA disease or MRSA carriage was based on clinical case data from the physician caring for the patient.

For additional analysis, the reported cases of MRSA were divided into the following categories: residents of Sweden traveling abroad for recreational purposes, residents of Sweden traveling abroad because of work or studies, foreign residents traveling to Sweden for a temporary visit, immigrants, internationally adopted children, or Swedish citizens returning home after having lived abroad for >1 year. The risk for MRSA in travelers, immigrants, and adopted children was also evaluated by including and excluding reported cases found through public health-initiated case-finding activities and secondary cases in transmission chains.

Swedish Tourist and Travel Database

Travel data from the commercial Swedish Tourist and Travel Database (TDB) were used as a denominator when analyzing the risk for being reported as having MRSA in travelers from Sweden. The TDB is based on monthly interviews with 2,000 randomly selected residents of Sweden. We used the TDB variables age group, sex, purpose of travel (leisure or work), main destination, and time of travel. TDB for 2000–2003 and interviews of 9,213 persons with a history of travel abroad were used. Information in the TDB was weighted and extrapolated to estimate the total number of travelers from Sweden and to derive a total of 45,855,000 travel episodes (*17*). Actual interviewees were used as controls in an adjusted model that estimated the odds ratio (OR) of having MRSA, and extrapolated estimates were used to calculate risks per 1,000,000 travelers. Because only persons <75 years of age were sampled for interviews for the TDB, 34 cases of MRSA in persons >74 years of age were excluded from the TDB analyses.

Data on Immigration and International Adoptions

Denominator data on the yearly number of immigrants to Sweden were obtained from Statistics Sweden (*18*). Included in the immigration figures were persons who received a residence permit because of family ties, European Economic Area convention, and labor permits; visiting students; and asylum seekers per country of citizenship. Data on the yearly number of international adoptions per country were obtained from the Swedish Intercountry Adoptions Authority (*19*).

Epidemiologic Typing of MRSA Isolates

Isolates from all persons with new cases of MRSA were sent to SMI, where laboratory diagnosis of MRSA was confirmed by using PCR for the *mecA* and the *nuc* genes (*20*). Epidemiologic typing of all isolates was performed by using pulsed-field gel electrophoresis (PFGE) according to the protocol of Murchan et al. (*21*). On the basis of PFGE results, representative isolates were selected for multilocus sequence typing (MLST) (*22*), and sequence types (STs) of isolates were determined by using the MLST database (www.mlst.net).

Statistical Analysis

Stata 8.2 software (StataCorp LP, College Station, TX, USA) was used for all statistical analyses, and a 95% level of significance was applied. Sex, age group, purpose of travel, and destination were used as explanatory variables in multivariable analysis of travelers with a diagnosis of MRSA and its reporting as an outcome. Generalized estimating equations with independent correlation and robust standard errors were used to enable clustering of data in the adjusted model that included all travelers <75 years of age.

Results

During 2000–2003, a total of 1,733 cases of MRSA were reported in Sweden, 444 of which were considered to have been acquired abroad. Among these imported cases, 292 (65.8%) were in residents of Sweden who traveled abroad; 31 (7.0%) had traveled for work reasons or studies. Fifty-six (12.6%) of the cases were in newly arrived immigrants to Sweden, 40 (9.0%) in internationally adopted children from non-Nordic countries, 30 (6.8%) in foreign residents visiting Sweden, 20 (4.5%) in citizens of Sweden returning home after having lived abroad for >1 year, and 6 (1.4%) in persons who could not be grouped. Of the imported MRSA cases, 246 (55.4%) were HA and 146 (32.9%) were CA; 190 (42.8%) were in persons with MRSA disease.

MRSA in Travelers Returning to Sweden

Of the 292 residents of Sweden who traveled abroad, 267 met the age criteria and were included in this analysis.

Most persons with MRSA had traveled to Thailand (n = 33), the United Kingdom (n = 24), Greece (n = 22), the former Yugoslavia (n = 20), Lebanon (n = 16), the United States (n = 14), Cyprus (n = 12), Spain (n = 12), Turkey (n = 9), and Syria (n = 8). The overall risk for being reported as having MRSA was 5.8 cases/1,000,000 travelers (95% confidence interval [CI] 5.2–6.6). The crude risk for travelers to specific regions ranged from 0.1 (95% CI 0.01–0. 4) per 1,000,000 travelers to Nordic countries to 59.4 (95% CI 44.5–79.3) per 1,000,000 travelers to North Africa and the Middle East (Table 1). Rank in risk for MRSA in the regions analyzed changed only slightly when we adjusted for confounders.

The adjusted OR for having MRSA was 1.5× higher among male participants (95% CI 1.2–2.0) than female participants (Table 1). The crude risk differed slightly between age groups and was highest among persons 60–74 years of age (11 cases/1,000,000 travelers; 95% CI 8.1–13.8), but no difference was found in the OR between age groups in the adjusted model. Work-related travel showed a lower crude risk for being reported as having MRSA, but when adjusting for other risk factors, the OR of MRSA was 2.4× higher among persons who traveled for work purposes than in leisure travelers (95% CI 1.5–3.8). To examine to what degree inclusion or exclusion of carriers would change our findings, analysis was repeated by using only the 133 (49.8%) cases in travelers with MRSA disease. We found only minor changes in ranking of ORs for geographic regions. The same 5 regions (Table 1) still ranked highest with higher ORs than other regions. The most prominent difference for ORs was that South America and Oceania and Pacific Islands changed places in rank.

Percentages of CA-MRSA for the respective regions, correlated linearly with adjusted ORs ($r = 0.81$, $p = 0.001$). Among age groups, the proportion of cases of CA-MRSA was highest in persons 0–14 years of age (83.3%), lowest in persons 60–74 years of age (9.3%), and intermediate for other age groups.

Table 1. Estimated number of travelers and cases of travel-associated acquisition of MRSA, Sweden, 2000–2003*

Variable	Estimated no. travelers	No. with MRSA infection (no. with MRSA disease)	Crude risk/1 million travelers (95% CI)	% Community-acquired cases	Adjusted OR (95% CI)
Sex					
F	25,219,000	109 (62)	4.3 (3.6–5.2)	42.2	Reference
M	20,634,000	158 (71)	7.7 (6.6–8.9)	27.2	1.5 (1.2–2.0)
Age group, y					
0–14	6,497,000	30 (24)	4.6 (3.2–6.6)	83.3	Reference
15–29	8,657,000	72 (36)	8.3 (6.6–10)	31.9	1.0 (0.6–1.5)
30–44	13,234,000	49 (26)	3.7 (2.8–4.9)	44.9	0.8 (0.5–1.3)
45–59	12,342,000	62 (29)	5.0 (3.9–6.4)	22.6	0.8 (0.5–1.2)
60–74	5,127,000	54 (18)	11 (8.1–13.8)	9.3	1.2 (0.7–1.9)
Purpose of travel					
Leisure	36,323,000	237 (123)	6.5 (5.7–7.4)	34.2	Reference
Work	9,532,000	30 (10)	3.1 (2.2–4.5)	26.7	2.4 (1.5–3.8)
Region of origin†					
Nordic	15,962,000	1 (0)	0.1 (0.01–0.4)	0.0	0.1 (0.01–0.6)
Western Europe	7,614,000	7 (3)	0.9 (0.4–1.9)	14.3	Reference
Southern Europe	7,601,000	18 (5)	2.4 (1.5–3.8)	0.0	2.4 (1.0–5.8)
Central and Eastern Europe	2,885,000	7 (5)	2.4 (1.2–5.1)	28.6	2.8 (1.0–8.1)
UK and Ireland	2,815,000	26 (11)	9.2 (6.3–13.6)	7.7	10.3 (4.4–24..0)
North America	1,627,000	17 (4)	10.4 (6.5–16.8)	35.3	10.6 (4.2–26.7)
Northeastern Mediterranean	4,317,000	63 (30)	14.6 (11.4–18.7)	38.1	15.8 (7.0–35.6)
South America	152,000	6 (5)	39.5 (17.7–87.9)	33.3	31.2 (10.0–97.6)
East Asia	1,622,000	54 (27)	33.3 (25.5–43.5)	27.8	36.5 (16.2–82.0)
Oceania and Pacific Islands	250,000	11 (5)	44.0 (24.4–79.5)	36.4	43.0 (15.5–119.4)
Sub-Saharan Africa	232,000	11 (7)	47.4 (26.3–85.6)	54.5	46.3 (17.3–123.6)
North Africa and Middle East	774,000	46 (31)	59.4 (44.5–79.3)	58.7	59.0 (25.1–138.9)
Total	45,855,000	267 (133)	5.8 (5.2–6.6)	33.3	

*MRSA, methicillin-resistant *Staphylococcus aureus*; CI, confidence interval; OR, odds ratio.
†Nordic: Denmark, Finland, Iceland, Norway; Western Europe: Austria, Belgium, France, Germany, Luxembourg, the Netherlands, Switzerland; Southern Europe: Italy, Malta, Monaco, Portugal, Spain; Central and Eastern Europe: Belarus, Bulgaria, Estonia, Hungary, Kazakhstan, Latvia, Lithuania, Poland, Romania, Russia, Slovakia, Czech Republic, Ukraine; North America: Canada, Cuba, Dominican Republic, Guadeloupe, Guatemala, Mexico, Panama, United States; Northeastern Mediterranean: Albania, Bosnia-Herzegovina, Cyprus, former Yugoslavia, Greece, Croatia, Macedonia, Serbia, Slovenia, Turkey; South America: Brazil, Chile, Colombia, Ecuador, Peru, Venezuela; East Asia: Afghanistan, Bangladesh, Cambodia, China, Hong Kong, India, Indonesia, Japan, South Korea, Malaysia, Maldives, Mongolia, Pakistan, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam, the Philippines; Oceania and Pacific islands: Australia, Hawaii, New Zealand, Samoa; Sub-Saharan Africa: Angola, Botswana, Chad, Ethiopia, the Gambia, Ghana, Kenya, Congo–Kinshasa, Madagascar, Senegal, Sierra Leone–Togo, Somalia, Sudan, Swaziland, South Africa, Tanzania–Uganda; North Africa and Middle East: Algeria, Egypt, Iraq, Iran, Israel, Jordan, Kuwait, Lebanon, Libya, Morocco, Saudi Arabia, Syria, Tunisia, United Arab Emirates.

MRSA in Immigrants

A total of 56 persons who had recently arrived in Sweden as immigrants had cases of MRSA (Table 2), giving an overall risk of 15.9 (95% CI 12.0– 20.7) cases/100,000 immigrants. MRSA disease was present in 27 (48.2%) of the immigrants. Twenty of the persons with MRSA came from the former Yugoslavia. The highest number of immigrants (n = 45,587) also came from this country; these persons had a risk of 43.8 (95% CI 26.8–67.8) MRSA cases/100,000 immigrants. Nearly as many immigrants came from Iraq, but the risk for MRSA was lower for this group (9.1, 95% CI 2.5–23.4 cases/100,000 immigrants).

MRSA in Adopted Children

In 2000–2003, a total of 4,169 children were adopted from non-Nordic countries. Forty adopted children from 12 countries were reported as having cases of MRSA shortly after their arrival in Sweden, i.e., 9.6 (95% CI 6.9–13.0) cases per 1,000 adoptions (Table 3). Of these adopted children with MRSA, 6 had MRSA disease. Risk for having MRSA ranged from 3.0/1,000 adoptions of children from Vietnam (95% CI 0.1–16.8) to 74.1/1,000 adoptions of children from the Philippines (95% CI 9.1–242.8). The largest number of adopted children (n = 1,074) came from the People's Republic of China. However, only 6.5/1,000 adoptees (95% CI 2.6–13.4) from this country had MRSA. Children adopted from South Korea showed 30.0 cases/1,000 adoptions (95% CI 16.5–49.8).

Type of MRSA and Geographic Region of Acquisition

STs inferred from PFGE results and region of acquisition for 414 MRSA cases (excluding secondary cases and cases with STs seen in <5 cases) are shown in the online Appendix Table (www.cdc/gov/EID/content/16/2/189-appT.htm). Also shown are the number of HA-MRSA and CA-MRSA cases per ST and region of acquisition. HA ST22 and ST36 predominated in cases from the United Kingdom and Ireland (15/33 and 11/33 MRSA cases, respectively). Among 92 cases from East Asia, the predominant MRSA were HA ST239 (n = 22 cases), CA ST30 (n = 13 cases), and CA ST72 (n = 6 cases). CA ST30 isolates were also seen in 5 cases from neighboring areas (3 from the Oceania and Pacific Islands region and 2 from Hawaii that belonged to North American region). Among the 50 cases from North Africa and the Middle East, CA ST80 (n = 18 cases) dominated. ST 80 was also isolated from 21 of 90 cases from the neighboring northeastern Mediterranean region. However, this region was mainly characterized by 30 HA ST239 (n = 30 cases). In cases from southern Europe, HA ST228 was most frequently seen (11/36 cases). Overall, STs 5, 22, 36, 125, 228, 239, and 241 were found most often in cases of HA-MRSA, and STs 30 and 80 were found most often in

Table 2. Imported cases of MRSA acquisition among immigrants, Sweden, 2000–2003*

Country of origin	No. immigrants†	No. with MRSA acquisition (no. with MRSA disease)	Risk/100,000 immigrants (95% CI)
Malta	21	1 (0)	4,761.9 (120.5–23,816.0)
Kazakhstan	933	4 (2)	428.7 (116.9–1,094.0)
South Africa	556	2 (1)	359.7 (43.6–1,293.3)
Sudan	360	1 (1)	277.8 (7.0–1,537.9)
Italy	1,382	2 (1)	144.7 (17.5–521.8)
Ecuador	995	1 (1)	100.5 (2.5–558.6)
Lebanon	2,100	2 (0)	95.2 (11.5–343.6)
Mongolia	1,055	1 (1)	94.8 (2.4–527.0)
Japan	1,274	1 (0)	78.5 (2.0–436.6)
The Philippines	1,335	1 (1)	74.9 (1.9–416.6)
Afghanistan	6,166	4 (3)	64.9 (17.7–166.0)
United States	4,094	2 (0)	48.9 (5.9–176.4)
Former Yugoslavia	45,587	20 (10)	43.8 (26.8–67.8)
Germany	6,564	2 (0)	30.5 (3.7–110.0)
Poland	3,658	1 (0)	27.3 (0.7–152.2)
Iran	7,856	2 (1)	25.5 (3.1–91.9)
Thailand	4,883	1 (0)	20.5 (0.5–114.0)
United Kingdom	5,306	1 (0)	18.9 (0.47.7–105.0)
Turkey	5,568	1 (1)	18.0 (0.5–100.0)
Russia	8,254	1 (0)	12.1 (0.3–67.5)
Somalia	8,546	1 (1)	11.7 (0.3–65.2)
Iraq	43,730	4 (3)	9.1 (2.5–23.4)
Other countries	191,302	0	–
Total	351,525	56 (27)	15.9 (12.0–20.7)

*MRSA, methicillin-resistant *Staphylococcus aureus*; CI, confidence interval.
†Included in the immigration figures were persons who received a residence permit because of family ties, European Economic Area convention, and labor permits; visiting students; and asylum seekers per country of citizenship.

Table 3. Imported cases of MRSA acquisition among children adopted from other countries, Sweden, 2000–2003*

Country	No. adoptions	No. with MRSA acquisition (no. with MRSA disease)	Risk/1,000 adoptions (95% CI)
The Philippines	27	2 (0)	74.1 (9.1–242.8)
South Korea	467	14 (0)	30.0 (16.5–49.8)
Bulgaria	91	2 (1)	22.0 (2.7–77.1)
Belarus	174	3 (1)	17.2 (3.6–49.6)
Russia	263	4 (0)	15.2 (4.2–38.5)
Ethiopia	71	1 (1)	14.1 (0.4–76.0)
Ukraine	74	1 (0)	13.5 (0.3–73.0)
People's Republic of China	1,074	7 (3)	6.5 (2.6–13.4)
Colombia	460	3 (0)	6.5 (1.3–18.9)
South Africa	154	1 (0)	6.5 (0.16–35.6)
India	243	1 (0)	4.1 (0.1–22.7)
Vietnam	328	1 (0)	3.0 (0.1–16.8)
Other countries	743	0	–
Total	4,169	40 (6)	9.6 (6.9–13.0)

*MRSA, methicillin-resistant *Staphylococcus aureus*; CI, confidence interval.

cases of CA-MRSA. Strains acquired in North America or European regions were associated with HA-MRSA.

Discussion

We have estimated risks for MRSA among travelers returning from specific geographic regions. Other studies have reported individual cases of MRSA diagnosed in connection with travel (*10*,*11*,*23*–*25*). Kaiser et al. (*12*) studied patients in the Netherlands who were repatriated after having received healthcare abroad, but found no association between country or region and MRSA diagnosis. However, the participation rate in their study was low, and the lack of statistically significant results could have been caused by the low number of cases.

The overall prevalence and transmission rate of MRSA within a country or region is just one of several factors that can influence the risk for MRSA acquisition by travelers to that country or region. This risk will also depend on type of exposure and duration of stay. For example, risk for MRSA acquisition will vary, depending on whether the person had received healthcare or had only community contacts, whether he or she stayed in a hotel at a holiday resort for a week, stayed with relatives for 3 months, or just stayed overnight on a business trip. In Europe, we observed a relatively high risk for MRSA acquisition in travelers to the United Kingdom, Ireland, and southern Europe, which is consistent with the high prevalence of MRSA reported for these regions (*26*–*28*). Other studies have shown that MRSA is hyperendemic to the Pacific region, East Asia, the Mediterranean region, and the Middle East (*27*–*29*). Thus, the higher risk for MRSA among travelers returning from these regions in our study may reflect such hyperendemicity. In addition, we found a higher risk for MRSA among travelers to sub-Saharan Africa and South America, and a risk for MRSA among travelers to central and eastern Europe that is comparable to that among travelers to southern Europe.

The risk for immigrants and adopted children of acquiring MRSA may not be representative of the overall risk in the general population in their respective countries of origin. These 2 populations may have spent time in institutional settings (e.g., orphanages, refugee camps, or hospitals) in which the risk for MRSA exposure may be different from that in the community in general. In addition, analysis of MRSA risk among newly adopted children and immigrants was based on small numbers of persons, which resulted in wide CIs of the estimated risks. Despite this uncertainty, agreement was seen between the estimated risk for MRSA acquisition among returning travelers, immigrants, and adopted children. Eight of 12 countries from which the adopted children with MRSA originated and 14 of the 22 countries from which immigrant cases originated belonged to the 6 regions with highest risk for MRSA among travelers (northeastern Mediterranean, South America, East Asia, Oceania and Pacific islands, sub-Saharan Africa, and North Africa and the Middle East).

Measuring the risk for MRSA in travelers, adopted children, and immigrants involved analyzing 3 different groups related to specified countries and regions. Because of the large degree of agreement between the 3 groups, results obtained may reflect not only risks for MRSA in these 3 populations, but also an overall comparatively higher risk for MRSA in some regions than in others.

The study was based on persons (carriers and those with clinical disease) with reported cases of MRSA in Sweden during 2000–2003. Only minor changes, which did not affect our primary findings, were seen when carrier cases were excluded from the travel analysis. Approximately 50% of MRSA cases among immigrants, but only 15% (6/40) of adopted children, had MRSA disease. Twelve of the 13 countries from which immigrants originated belonged to the 6 regions with highest risk for MRSA among travelers. For adopted children, the numbers of countries

involved and cases decreased when carriers were excluded. However, 2 of 4 countries (4 of 6 cases) still belonged to the same 6 regions. Results for these 3 populations with acquired MRSA are consistent even when carriers were excluded from the analysis.

One strength of our study is that all regions/countries were investigated by using the same surveillance system, thus avoiding problems that might arise when data for different countries are compiled from different sources. Because the travel analysis was based on residents of Sweden who traveled to other countries and results were adjusted for sex and age, the main conclusions should also be valid for other western countries. Possible biases related to MRSA cases reported in Sweden during 2000–2003 and use of the TDB have been evaluated (*16*,*30*). Information from the TDB has shown good agreement with data from other sources (*31*). We could not control for length of stay of travelers at their destination because such information for MRSA cases was not available. However, persons with cases of MRSA who traveled for work purposes were commonly employed at the destination for a few months, rather than having been on short business trips.

Most persons infected with MRSA had HA-MRSA; only 33% had CA-MRSA. However, CA-MRSA showed a strong correlation with regions with a high risk for MRSA among travelers. This finding might be caused by a higher propensity among clinicians to sample patients with a history of activity (e.g., backpacking) under less than optimal sanitary conditions or travel to exotic regions. The 5 regions with the highest OR of acquiring MRSA in adjusted analysis were outside Europe.

Geographic distribution of MRSA STs was consistent with that in studies of other regions. ST22 and ST36 are well documented in the healthcare system in the United Kingdom (*32*). ST80 is common in the Mediterranean and Balkan region, the Middle East, and several countries in Europe, and epidemiologically related to persons with direct or indirect contact with the Mediterranean and Middle East (*1*,*11*,*12*,*23*–*25*). ST239 is common Asia (*33*,*34*) and the northeastern Mediterranean (*35*); ST30 is common in the Pacific region, East Asia, and Oceania (*1*,*36*); and ST228 is common in southern Europe and Germany (*37*,*38*).

In addition to showing an association between specific STs and regions, we also identified the transmission setting (healthcare or community) in which the isolate was acquired. We observed that most STs among imported cases of MRSA were associated with either HA-MRSA or CA-MRSA. ST30 and ST80 were strongly associated with CA-MRSA. ST72 was identified only among 8 adopted children from South Korea. However, information for the orphanages was not available. Thus, the possibility that 8 children could have been part of the same local transmission chain could not be ruled out. However, ST72 has recently been reported from several areas of South Korea among children and as a community-associated sequence type (*39*,*40*).

Several limitations of our study involve factors relevant to MRSA acquisition (length of stay abroad, contacts with institutions with a higher probability of MRSA transmission such as healthcare settings, orphanages, or refugee camps) that were not measured. In addition, there may have been inconsistencies in how MRSA cases were selected in the surveillance system. In some counties in Sweden, MRSA screening of adopted children is conducted; in other counties, it is not practiced. Thus, underdiagnosis of MRSA carriage among adopted children was likely. No similar screening recommendations are known for newly arrived refugees or immigrants.

In countries with a low prevalence of MRSA, a larger proportion of cases are imported than in countries with a high prevalence of MRSA. The risk for additional domestic transmission indicates a greater need to focus public health measures toward these imported cases. We observed differences in risk for MRSA among travelers returning from different regions, adopted children, and immigrants. Risk estimates for MRSA measured in these 3 groups showed a consistent pattern; some regions showed a higher risk for acquiring MRSA. Although most imported cases of MRSA were HA-MRSA, community acquisition showed a correlation with regions that have a high risk for MRSA among travelers. Knowledge of these differences in risk for MRSA will improve control measures and decrease domestic transmission.

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Risk Factors for and Estimated Incidence of Community-associated *Clostridium difficile* Infection, North Carolina, USA[1]

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Specify the prevalence of community-acquired *Clostridium difficile* infection
- Describe demographic trends in community-acquired *C. difficile* infection
- Identify case characteristics of *C. difficile* infection
- List risk factors for community-acquired *C. difficile* infection

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We determined estimated incidence of and risk factors for community-associated *Clostridium difficile* infection (CA-CDI) among patients treated at 6 North Carolina hospitals. CA-CDI case-patients were defined as adults (>18 years of age) with a positive stool test result for *C. difficile* toxin and no hospitalization within the prior 8 weeks. CA-CDI incidence was 21 and 46 per 100,000 person-years in Veterans Affairs (VA) outpatients and Durham County populations, respectively. VA case-patients were more likely than controls to have received antimicrobial drugs (adjusted odds ratio [aOR] 17.8, 95% confidence interval [CI] 6.6–48] and to have had a recent outpatient visit (aOR 5.1, 95% CI 1.5–17.9). County case-patients were more likely than controls to have received antimicrobial drugs (aOR 9.1, 95% CI 2.9–28.9), to have gastroesophageal reflux disease (aOR 11.2, 95% CI 1.9–64.2), and to have cardiac failure (aOR 3.8, 95% CI 1.1–13.7). Risk factors for CA-CDI overlap with those for healthcare-associated infection.

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Clostridium difficile is an anaerobic spore-forming gram-positive bacillus that produces exotoxins that are pathogenic to humans. *C. difficile* is known to infect persons receiving antimicrobial drug therapy, older and severely ill patients who are hospitalized, or residents of long-term care facilities. *C. difficile* infection (CDI) is manifested as as diarrhea, pseudomembranous colitis, and, occasionally, toxic megacolon or even death. Recent reports suggest an increasing incidence and severity of CDI (*1*–*3*) that may be related to the emergence of a hypervirulent strain (*4*–*6*). In addition, reports have been published of CDI emerging in persons previously thought to be at low risk, including otherwise healthy persons in the community (*7*–*9*).

Community-associated or -acquired CDI (CA-CDI) was first reported in 1984 by Stergachis et al. (*10*) who found that the attack rate of CA antimicrobial drug–associated colitis requiring hospitalization was 1.4/100,000 population. In a review by Riley et al. (*11*) of 580 *C. difficile* toxin–positive stool samples submitted from patients with diarrhea and a clear history of recent (≤4 weeks) antimicrobial drug exposure, 10.7% were from patients who did not have a recent history of hospitalization. Additional reports indicated incidences from 7.7–12.2 cases of CA-CDI per 100,000 persons in US communities (*7*,*12*) to 25 primary episodes per 100,000 persons per year in Sweden (*8*). In December 2005, the US Centers for Disease Control and Prevention (CDC) reported a series of severe CDI cases in populations previously considered at low risk, including generally healthy persons living in the Philadelphia, Pennsylvania, USA, area (*13*).

In January 2006, CDC was notified by a regional Veterans Affairs (VA) hospital in North Carolina that surveillance conducted from October 2004 through December 2005 indicated that 35% of patients with CDI experienced onset of disease in the community. Fewer than 10% of these patients had stayed overnight in a healthcare facility, and only half had received antimicrobial drugs in the previous 2 months (*14*). In response to apparent emerging CDI in the community, CDC was invited by the North Carolina Department of Health and Human Services to investigate these findings and to recommend control measures.

We retrospectively identified cases of CA-CDI in 4 North Carolina VA hospitals and all hospital catchments in Durham County, North Carolina, to determine the estimated incidence in each population. In addition, we assessed risk factors for CA *C. difficile* disease using case–control methods.

Methods

Settings and Case Finding

Data were collected from 6 North Carolina hospitals, including the Durham VA hospital, 2 other local hospitals, and 3 other VA hospitals located throughout the state. This activity was determined by CDC not to be human subject research; thus, human subject regulations did not apply.

Case finding involved reviewing positive *C. difficile* toxin assays from the VA infection control database, Computer Patient Record Systems, and the surveillance database of the Duke University Hospital network. Medical and laboratory records were retrospectively reviewed for case-patients from January through December 2005. Trained coders abstracted data from electronic and paper medical records using standardized forms. Data collected included demographics, signs and symptoms at the time of admission, laboratory results, prior hospitalization, risk factors, treatments, and outcomes.

Case Definitions

A CDI case-patient was defined as a person ≥18 years of age with a nonformed (i.e., taking the shape of the container) stool specimen with positive test results for *C. difficile* toxin. If the case-patient had a previous *C. difficile*–positive stool test results within the 8 weeks preceding the collection date, he or she was excluded. All laboratories used the same toxin enzyme immunoassay (C. DIFFICILE TOX A/B II; TECHLAB, Blacksburg, VA, USA). CDI cases were further categorized according to when and where the stool specimen was collected, as community onset, CA, or inpatient healthcare exposure. We defined community onset as occurring 1) in an outpatient setting; 2) ≤3 calendar days after hospital admission; or 3) 4–5 calendar days after admission and with diarrhea documented <3 days after hospital admission. CA-CDI was defined as community-onset CDI for which there was no inpatient healthcare exposure within 8 weeks before the stool collection date. Inpatient healthcare exposure was defined as admission to an acute-care hospital or long-term care facility that provided skilled nursing care to the patient for ≥1 overnight stay.

We excluded CDI case-patients whose medical records appeared incomplete for determination of disease categorization; these case-patients were classified as unknowns. Community-onset CDI case-patients who were seen at Durham County hospitals but who resided outside the state were excluded because of frequent gaps in data necessary to determine the date of their last discharge from a healthcare facility and because their data could not contribute to the county population incidence. Among CA-CDI case-patients, ambulatory patients with a history of bone marrow transplant (BMT) or end-stage renal disease (ESRD) were excluded because of their intensive outpatient healthcare exposures. In addition, all prisoners were excluded. Although included in the incidence estimation, patients with inflammatory bowel disease and chronic diarrhea were excluded from the case–control study because they are rec-

ognized populations at increased risk for CDI and because their disease symptoms are difficult to differentiate from the symptoms of CDI (*15–17*).

We interviewed a subset of case-patients to confirm and expand information available from medical records pertaining to previous inpatient healthcare exposures and medication histories. We also surveyed physicians in Durham County to assess perceptions regarding the frequency and severity of CDI in the community and to determine laboratories used for *C. difficile* diagnostic testing. This survey included physicians working in emergency departments and in family medicine, internal medicine, obstetrics and gynecology, infectious diseases, gastroenterology, and urgent care practices. We also contacted other academic institutions and laboratories in nearby counties to determine whether our case finding was comprehensive.

VA Case–Control Study

We conducted an unmatched 1:3 case–control study of VA CA-CDI case-patients using controls chosen from among VA outpatients randomly selected from VA outpatient clinics seen at the 4 facilities on 4 random dates distributed throughout 2005. Exclusion criteria for controls included a documented clinical diagnosis of diarrhea or a stool test result positive for *C. difficile* toxin in 2005, a history of inpatient healthcare exposure in the prior 8 weeks, or a history of ESRD. Similar data were collected for the controls as for case-patients, except for prior hospitalization within 8 weeks.

We attempted phone interviews of all the VA CA-CDI case-patients, but not the controls, regarding their admission, symptoms, and medications. To overcome the limitation of recall bias, we reviewed electronic records after asking case-patients if they received their medications only from the VA (where electronic records were then reviewed) or whether they ever (also) obtained prescriptions from non-VA providers. All case-patient interviews were completed by the first quarter of 2006.

Durham County Case–Control Study

We conducted a case–control study at the 2 major hospitals serving Durham County residents. Controls were randomly selected from the county voter registration list; we made 3 attempts to reach residents by phone and elicit their participation. Exclusion criteria for controls included a history of inpatient healthcare exposure or diarrhea in the year 2005, ESRD, or BMT. We also performed phone interviews on a convenience sample of case-patients. All interviews were completed by December 2006.

Data Analysis

Data for the case characterization and case–control study were entered into Microsoft Office Access 2003 (Microsoft Corp., Redmond, WA, USA). Data checks were performed and double entries were removed. Incidence was estimated for the North Carolina VA hospital catchments by dividing the number of CA-CDI case-patients by all veterans registered for outpatient services at the 4 VA facilities in 2005 per 100,000 person-years. An estimate of the Durham County population-based rate was determined by dividing the number of CA-CDI case-patients from Durham County (including VA case-patients who resided in the county) by the 2005 adult (>18 years of age) population census per 100,000 person-years.

Multivariable analysis was performed by using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and stepwise logistic regression. Significant variables based on the univariate analysis ($p<0.05$) were included in the multivariable model. We used the Hosmer and Lemeshow and the residual χ^2 goodness-of-fit tests. We estimated adjusted prevalence odds ratios (aORs) with 95% confidence intervals (CIs) from the regression procedure.

Results

Of the 1,046 CDI case-patients identified from the 6 study facilities from January through December 2005, 426 (40.7%) were community-onset (Figure). A total of 214 community-onset CDI case-patients were excluded; 94 had inpatient healthcare exposures within the 8 weeks prior to positive toxin assay, 29 had ESRD, 20 were from out of state, 17 were BMT patients, 8 were prisoners, and 46 had an unknown status regarding prior inpatient healthcare exposures. The 212 case-patients with CA-CDI represented

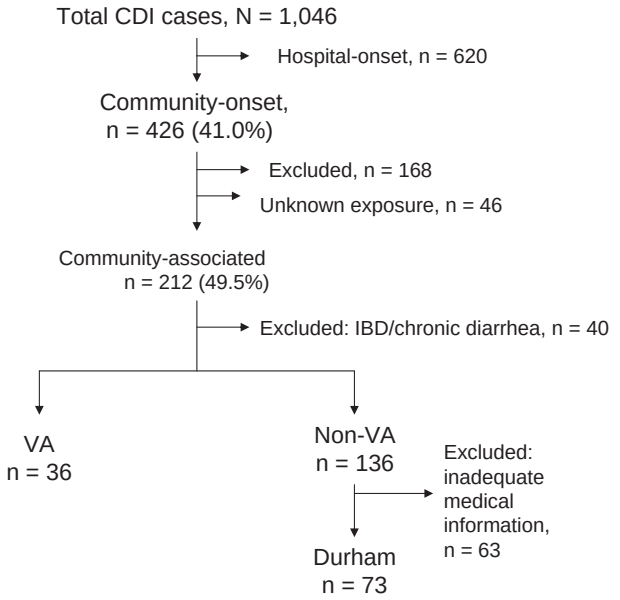

Figure. Categorization of *Clostridium difficile* infection (CDI) cases from 6 hospitals, North Carolina, 2005. IBD, irritable bowel disease; VA, Veterans Affairs hospital.

49.5% of all cases of community-onset CDI and 20% of overall cases of CDI.

The estimated incidence of CA-CDI in the VA catchments and county adult population was 21 and 46 per 100,000 persons per year, respectively (Table 1). Among men in the VA and in Durham County, those 45–64 years of age had a higher incidence than those 18–44 and >64 years of age combined ($p<0.05$). Overall, incidence was higher among county female case-patients than male case-patients (62 vs. 28/100,000 persons per year, respectively; $p = 0.0005$).

Table 1. Estimated incidence of community-associated *Clostridium difficile* infection per 100,000 persons among VA and Durham County populations, North Carolina, USA, 2005*

Population	Age group, y, no. cases			Overall
	18–44	45–64	≥65	
VA				
M	14.7	28.5†	15.9	20.8
Durham County				
M	11.0	56.6†	57.5	28.4
F	21.9	70.4	204.6	61.9‡
Overall	16.5	63.9	146.4	46.0

*VA, Veterans Affairs.
†$p<0.05$ when compared with age groups 18–44 and ≥65 y combined.
‡$p<0.005$ when compared with men from Durham County.

Characteristics of CA-CDI Cases

A total of 109 CA-CDI case-patients were found among VA catchments or Durham County residents for whom full case-characteristics were available after exclusion criteria (Figure). The characteristics of CA-CDI case-patients are described in the online Appendix Table (www.cdc.gov/EID/content/16/2/197-appT.htm). We did not compare the 2 populations.

Nineteen case-patients in the VA catchments and 12 case-patients from Durham County had histories of hospitalization >8 weeks prior to symptom onset; medians were 33 weeks (range 10–84) and 34 weeks (range 9–92), respectively. The remaining case-patients had no recorded history of previous hospitalization. Median time from symptom onset to testing was 1 week (range 1 day–9 weeks).

Overall, 58% of case-patients in each population were admitted around the time of their CDI diagnosis, for a median duration of 1 week (maximum 3 and 5 weeks, Durham County and VA catchments, respectively). Diarrhea was the most common sign or symptom, followed by abdominal pain and vomiting. Among potential exposures, >50% of case-patients in each population had ≥1 outpatient visit in the 3 months before the test date. Overall, 53 case-patients (49%) did not have exposure to antimicrobial drugs in the 3 months prior to the test date. Among those who had recently received antimicrobial drug therapy, pencillins and quinolones were most commonly reported.

Case-patient Interview

We interviewed 22 VA and 31 county (non-VA hospital) resident CA-CDI case-patients. Only 2 (4%) of the 53 patients interviewed were reclassified as other than CA-CDI on the basis of interview findings: 1 had a history of CDI 2 months before the index episode, and 1 was newly identified as an ESRD patient. Of the remaining interviewees, 25 were able to provide information on whether they had been exposed to antimicrobial drugs. Of the 11 interviewees whose medical records suggested no exposure to antimicrobial drugs within the past 3 months, 5 (45%) reported taking antimicrobial drugs during this time. These included 3 from the VA who indicated that they received all their medications from the VA; however, their electronic medical records did not have documentation of recent antimicrobial drug prescriptions.

Case–Control Study at VA

Case-patients and controls were similar with regard to age, sex, and race (Table 2). Over the 3 months before CDI onset, exposure to antimicrobial drugs (OR 19.6, 95% CI 7.6–51, $p<0.0001$), antimotility medications (OR 6.6, 95% CI 1.2–37.8, $p<0.05$), and outpatient visits (OR 6.1, 95% CI 2–18.6, $p<0.05$) were most strongly associated with case status. Among antimicrobial drugs, exposure to penicillins and quinolones were significant. In the multivariable analysis, antimicrobial drug use with an aOR of 17.8 (95% CI 6.6–48, $p<0.0001$) and outpatient visits (aOR 5.1, 95% CI 1.5–17.9, $p<0.05$) were significant risk factors.

Case–Control Study in Durham County

Information was obtained for all 73 case-patients but only for 48 controls (Table 3). The case-patients and controls were similar with regard to age, sex, and race. Among potential exposures during the previous 3 months, exposure to antimicrobial drugs (OR 3.9, 95% CI 1.6–0.5, $p<0.05$) was most strongly associated with case status. In the multivariable analysis, antimicrobial drug use (aOR 9.1, 95% CI 2.9–28.9, $p<0.05$), gastroesophageal reflux disease (GERD) (aOR 11.2, 95% CI 1.9–64.2, $p<0.05$), and cardiac failure (aOR 3.8, 95% CI 1.1–13.7, $p<0.05$) were independent risk factors.

Physician Survey

Of 33 Durham County primary care and relevant specialty medicine practices surveyed, 59 physicians from 16 (48.5%) practices responded. Only 2 commercial laboratories, in addition to the 2 Durham hospital laboratories, were used for diagnostic testing of *C. difficile* in the outpatient setting. One laboratory was unable to provide any information regarding positive *C. difficil*e tests performed on persons living in Durham County, whereas the other laboratory had 14 positive *C. difficile* test results for this population

Table 2. Results of community-associated *Clostridium difficile* infection case–control study, Veterans Affairs, North Carolina, USA, 2005*

Characteristic	No. (%) persons		OR (95% CI)
	Case-patients, n = 36	Controls, n = 108	
Demographics			
Median age, y (range)	62 (38–85)	64 (36–86)	
Female gender	4 (11)	4 (4)	
Non-Hispanic white race	25 (69)	70 (65)	
Coexisting health conditions			
Hypertension	16 (44)	75 (69)	0.35 (0.2–0.8)†
Cardiac failure	6 (17)	5 (5)	4.1 (1.2–14.4)†
Exposures‡			
Outpatient visit	32 (89)	69 (57)	6.1 (2.0–18.6)†
Antimicrobial drugs	24 (66)	10 (9)	19.6 (7.6–51.0)†
Penicillins	13 (36)	3 (3)	19.8 (5.2–75.1)§
Quinolones	6 (17)	3 (3)	7 (1.7–29.7)†
NSAIDs	6 (17)	30 (28)	0.5 (0.2–0.4)
Antimotility medications	4 (11)	2 (2)	6.6 (1.2–37.8)†
Gastric acid suppressors	18 (50)	37 (34)	1.9 (0.9–4.1)
H2 blockers	7 (19)	13 (12)	1.8 (0.6–4.8)
Proton pump inhibitors	13 (36)	26 (24)	1.7 (0.7–4.0)
Steroids	4 (11)	3 (3)	4.4 (0.9–20.5)
Multivariable analysis¶			
Antimicrobial drugs			17.8 (6.6–48.0)#
Outpatient visit			5.1 (1.5–17.9)†#

*OR, odds ratio; CI, confidence interval; NSAIDs, nonsteroidal antiinflammatory drugs.
†p<0.05.
‡Exposures among case-patients and controls were within 3 months prior to the test date.
§p< 0.0001.
¶Goodness-of-fit tests: residual χ^2, p = 0.33; Hosmer and Lemeshow, p = 0.12.
#Adjusted OR.

during 2005. Additional information about these cases was unavailable.

Discussion

We found that 20% of all CDI cases were CA, similar to the 22%–28% found in previous studies conducted in Sweden (*8*,*18*). We are not aware of previous studies from the United States that determined the proportion of CA-CDI from all CDI cases. However, available literature on the incidence of CA-CDI in the United States (*7*,*13*,*19*) reports lower rates than what we found in the VA (21/100,000 persons/year) and Durham County (46/100,000 persons/year) catchments. The annual incidence in Philadelphia in 2005 (7.6/100,000 persons/year) and the incidence in Connecticut in 2006 (6.9/100,000 persons/year) were based on passive, voluntary reports of CA-CDI in mostly healthy persons and thus likely underestimated the true incidence and spectrum of disease (*13*,*19*). However, a recent active case-finding study for CA-CDI conducted in the United Kingdom (*20*) suggested rates of 20–30 per 100,000, similar to the studies conducted in Sweden (*8*,*18*). Furthermore, a recent study from Manitoba, Canada, found that ≈20% of CDI cases were CA, and annual incidence was ≈20/100,000 (*21*). Apparent differences in the incidence of CA-CDI likely reflect differences both in study approaches and population characteristics. Nonetheless, CA-CDI should be considered a serious public health concern in need of further understanding and improved surveillance. Although it is unclear why the estimated incidence rates in Durham County are the highest for CA-CDI reported, recent evidence indicate that the rates in the county may have since declined (*22*), a phenomenon anecdotally experienced in other locations in the southeastern United States (R.P. Gaynes, pers. comm.) (*23*).

The incidence rate we found was highest for the 45–64 years of age category (28.5/100,000) for the VA catchments. In Durham County, men 45–64 years of age had a higher rate than other those in age categories combined (p<0.05). This finding is notable because CDI is usually associated with older patients (*9*,*19*). Women had a higher rate than males in the community (62 vs. 28 cases/100,000 persons/year, respectively; p = 0.0005), as was also recently noted in a report from Connecticut (*19*). Although some data suggest an increased risk for multiple CDI recurrences in healthy women (*24*) and recent reports have noted severe CDI in pregnant women (*25*), female gender has not been previously a well-documented risk factor for CDI.

Previous reports have shown that an age >65 years is a risk factor for hospital-onset CDI (*2*,*26*,*27*). In comparison, the median age of the case-patients in this community appeared to be younger, 61 years in Durham County and

Table 3. Results of community-associated *Clostridium difficile* infection case–control study, Durham County, North Carolina, USA, 2005*

Characteristics	No. (%) persons		OR (95% CI)
	Case-patients, n = 73	Controls, n = 48	
Demographics			
Median age, y (range)	61 (20–101)	55 (22–87)	
Female gender	57 (58)	34 (71)	
Non-Hispanic white race	34 (47)	35 (73)	0.32 (0.12–0.7)†
Coexisting health conditions			
Cardiac failure	20 (27)	5 (10)	3.24 (1.12–9.4)†
GERD	20 (27)	2 (4)	8.7 (1.9–39.1)†
Hypertension	36 (49)	13 (27)	2.6 (1.2–5.7)†
Exposures‡			
Antimicrobial drugs	32 (44)	8 (17)	3.9 (1.6–9.5)†
NSAIDs	13 (17.8)	24 (50)	0.2 (0.1–0.5)†
Gastric acid suppressors	15 (21)	5 (10)	2.2 (0.75–6.6)
H2 blockers	6 (8)	3 (6)	1.3 (0.3–5.6)
Proton pump inhibitor	9 (12)	2 (4)	3.2 (0.7–15.7)
GERD	20 (27)	2 (4)	8.7 (1.9–39.1)†
Multivariable analysis§			
Antimicrobial drugs			9.1 (2.9–28.9)†¶
NSAIDs			0.2 (0.1–0.5)†¶
GERD			11.2 (1.9–64.2)†¶
White race			0.2 (0.05–0.40)†¶
Cardiac failure			3.8 (1.1–13.7)†¶

*OR, odds ratio; CI, confidence interval; GERD, gastroesophageal reflux disease; NSAIDs, nonsteroidal antiinflammatory drugs.
†p<0.05.
‡Exposures among case-patients were within 3 months prior to the test date; exposures for controls were for all of 2005.
§Goodness-of-fit tests: residual χ^2, p = 0.38; Hosmer and Lemeshow, p = 0.77.
¶Adjusted OR.

63 years in VA. In addition, the proportion and severity of fever, leukocytosis, and renal insufficiency for our case-patients were lower (*26*,*28*,*29*). No case-patients were admitted to the intensive care unit for CA-CDI, and none underwent colectomy. One case-patient in each population died within 10 days of diagnosis, and the death was attributable to CDI. Nonetheless, 15% had disease severe enough to require a visit to the emergency department, and another 59% required hospital admission for CDI management.

Antimicrobial drug exposure has long been known as a risk factor for healthcare-associated CDI. However, among CA cases in our study, 49% were not exposed to antimicrobial drugs. This percentage was slightly higher than the recent findings from Philadelphia (*13*) and Connecticut (*19*), where 24% and 36%, respectively, were not exposed to antimicrobial drugs. In contrast, Dial et al. found the absence of antimicrobial drug exposure >90 days to range from 60% to 70% (*30*). However, their analysis was limited to a clinical research database in which some hospitalization and antimicrobial drug exposures may not have been included. Two prospective studies have recently been conducted in the community. Bauer et al. (*31*) found that 42% of CA-CDI case-patients had not been exposed to antimicrobial drugs during the prior 6 months, and Wilcox et al. (*20*) found that 84% case-patients had not received antimicrobial drugs during the month before *C. difficile* detection. One hypothesis to explain the absence of antimicrobial drug exposure is that there are unmeasured factors affecting the epidemiology of CDI. For example, remote antimicrobial drug exposure, or exposure to other medications with antimicrobial activity, may be increasing the risk of disease; alternatively, increased awareness of CDI may be leading to increased testing and documentation of *C. difficile* in patients not previously tested. Another possibility is that strains with new virulence properties (e.g., binary toxin) that enable disease in the absence of prior antimicrobial drug use have emerged. Despite antimicrobial drug exposure being absent in many patients, we found that this exposure remained the most important modifiable risk factor for CA-CDI.

Additional risk factors included markers of chronic disease such as outpatient visits in the VA population and GERD and cardiac failure in the county population. In the VA population, frequent outpatient visits could reflect transmission in ambulatory care settings and could be a marker of a more severe underlying disease. Unlike other recent studies (*9*), we did not find proton pump inhibitors or H2 blockers were a major risk factor for CA-CDI. However, the finding of GERD as a risk factor suggests the possibility that undocumented use of over-the-counter proton pump inhibitors could have increased risk in these patients. Alternatively, there may be factors in the pathogenesis of

GERD that increases the risk for CDI.

Our study has several limitations. First, it is likely that there was incomplete case ascertainment, especially in those who underwent testing by outside laboratories so that, as high as these population incidence estimates are, they are likely underestimates of the true incidence. However the degree of underestimate is less likely in the VA system as there is financial incentive for patients to undergo testing within the system. The community physician survey conducted in Durham County indicated that there were 2 commercial laboratories other than Durham hospital laboratories used for testing. Although we were unable to determine the number of *C. difficile* tests performed at 1 laboratory, only 14 case-patients were identified from the other. It is also possible that patients received empiric therapy for CDI without a test being performed. However, the same survey of Durham County physicians indicated this was not a common practice. Some potential case-patients were categorized as unknowns when little or no medical records were available. We did not collect data on laboratory testing performed for any other enteric pathogens besides *C. difficile* nor did we perform cultures for *C. difficile,* and therefore no isolates were available.

Instead, case confirmation was limited to toxin immunoassay testing using the C. DIFFICILE TOX A/B II TECHLAB test. In an independent review (*32*), the sensitivity of this test was 83.3% and the specificity was 98.7%. To address the concern of inadequate sensitivity in the toxin immunoassay and to avoid any misclassification bias in our case–control studies, we excluded controls who had diarrhea. Despite the high specificity of this test, there are valid concerns that if a low-prevalence population, such as relatively asymptomatic persons without prior antimicrobial drug exposures, is tested, the likelihood of a false-positive result may be unacceptably high. Although this is an insurmountable obstacle to a retrospective analysis of current clinical testing practice for CDI, the fact that all study laboratories had rejection criteria to prevent testing formed stool, near uniform medical record documentation of diarrhea (i.e., patients with documented absence of diarrhea were excluded), and a median duration of diarrhea symptoms of 1 week suggests a reasonable pretest likelihood of CDI among these patients.

Another limitation was that few interviews were performed with case-patients. However, the adequacy of records indicating exposure to inpatient healthcare or antimicrobial drugs was verified among 53 case-patients who were interviewed by telephone. Only 1 case-patient was reclassified on the basis of an undocumented healthcare exposure, which was discovered during the interview process. Five of the 11 case-patients for whom antimicrobial drug exposure was not identified in their available medical records reported antimicrobial drug use. However, 3 of these were VA case-patients for whom medical records did not document such use, which suggests that some patients may have been mistaken about their antimicrobial drug exposure.

Another limitation is that our use of outpatient controls for the VA case–control study may have resulted in bias toward the null with regard to outpatient healthcare-related risk factors. Although we attempted to contact >400 candidate controls from the voter registration list for the Durham County case–control study, we encountered difficulty in reaching persons by phone and eliciting their participation. This resulted in only 48 controls being available and limited the power of this analysis.

In summary, CA-CDI is a relatively common clinical diagnosis. Although we did not determine the incidence in children, we found that CA-CDI in Durham County has a spectrum of disease that involves predominantly middle and older-aged women with underlying illness. As previously documented in other recent studies, this disease may, and commonly does, occur in patients without recent antimicrobial drug exposure. Nonetheless, antimicrobial drug exposure use remains the most important modifiable risk factor, suggesting prudent antimicrobial use remains a prominent public health prevention strategy. Further research into the incidence, sources, and risk factors for CA-CDI should be an ongoing public health priority.

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Household Responses to Pandemic (H1N1) 2009–related School Closures, Perth, Western Australia

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School closure is often purported to reduce influenza transmission, but little is known about its effect on families. We surveyed families affected by pandemic (H1N1) 2009–related school closures in Perth, Western Australia, Australia. Surveys were returned for 233 (58%) of 402 students. School closure was deemed appropriate by 110 parents (47%); however, 91 (45%) parents of 202 asymptomatic students reported taking ≥1 day off work to care for their child, and 71 (35%) had to make childcare arrangements because of the class closures. During the week, 172 (74%) students participated in activities outside the home on ≥1 occasion, resulting in an average of 3.7 out-of-home activities for each student. In our survey, activities outside the home were commonly reported by students affected by school closure, the effect on families was substantial, and parental opinion regarding school closures as a means to mitigate the outbreak of pandemic (H1N1) 2009 was divided.

On Friday, June 5, 2009, the Western Australia Department of Health received its third notification of confirmed infection with influenza A pandemic (H1N1) 2009. The patient was an elementary school student from Perth, Western Australia, Australia, who had recently returned from a sporting club excursion to Victoria, another Australian state, which had already experienced >600 cumulative confirmed cases of pandemic influenza (*1*).

Over the next 3 days (June 6–8), vigorous contact tracing and testing confirmed 11 more pandemic (H1N1) 2009 infections among schoolchildren; all had either visited Victoria or were close contacts of confirmed case-patients who had traveled to Victoria. These 11 children attended 3 schools located within 2 km of each other in a socioeconomically advantaged area of Western Australia's capital, Perth, which has a population of 1.7 million (*2*). On Sunday, June 7, in accordance with Australian public health practice at the time, the Department of Health advised the 3 schools to cancel classes for the coming week. School A, a public school, closed entirely; schools B and C, both private, cancelled classes for grade 5 and grades 5–7, respectively. The grades closed at schools B and C were those in which at least 1 student was confirmed as having pandemic (H1N1) 2009 virus infection.

School closure (i.e., either closure of school or dismissal of classes) is a nonpharmaceutical intervention often recommended for mitigating virus transmission during an influenza pandemic (*3*,*4*). However, little is known about the effect of school closures on students and families. We describe the activities of students affected by school closure, the effect of school closure on families, and parental opinions regarding school closures implemented in response to influenza A pandemic (H1N1) 2009.

Methods

Parents of all students excluded from attendance at schools A, B, and C were surveyed to ascertain the age of their child; the onset of illness, if any, in their child during the school closure period; the need for special childcare arrangements due to the closure; whether the child went out of the home during the school closure period; and parental perspectives on the consequences and appropriateness of the school closure. Parents were asked to complete a written questionnaire for their child; no personal identifiers

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were obtained. Surveys were distributed by schools on June 22, 2009 (10 days after school closure ended), and collected on July 3, 2009.

A case-patient was defined as a student with PCR results positive for influenza A pandemic (H1N1) 2009 virus. A contact was defined as a student who had been in a classroom with a case-patient for ≥4 hours or who had had another period of close physical proximity (e.g., sitting within 1 m of the case-patient for at least 15 min) during the case-patient's infectious period (i.e., 1 day before until 7 days after symptom onset). Other students affected by the closure but who did not meet case-patient or contact criteria were defined as school peers.

Influenza-like illness (ILI) was defined as an illness with fever and cough and/or sore throat. Upper respiratory infection (URI) was defined as an illness not meeting ILI criteria but exhibiting ≥1 of the following signs or symptoms: sore throat, cough or runny nose. Asymptomatic students were defined as contacts and peers in whom ILI or URI did not develop during the period of school closure. Ill students were defined as case-patients, contacts, and peers who developed ILI or URI during the school closure period.

The school closure period was defined as the interval from the first day classes were cancelled to the first day classes resumed (i.e., June 8–14, 2009) (Figure 1). Special childcare arrangements were defined as childcare activities other than the students caring for themselves at home or care provided by an adult member of the household. To separate the effects of the school closure by itself from the consequences of caring for a symptomatic child, the analysis of parental time off work and special childcare arrangements was limited to parents of asymptomatic students.

Frequencies, means, proportions, and Kruskal-Wallis tests of significance were calculated by using Epi Info 2000 (Centers for Disease Control and Prevention, Atlanta, GA, USA; www.cdc.gov/epiinfo/epiinfo.htm). Pearson χ^2 statistics were obtained by using SPSS version 17.0 software (SPPS Inc., Chicago, IL, USA).

Results

Surveys were returned for 233 (58%) of the 402 students affected by school closure; 49%, 59%, and 67% of surveys from schools A, B, and C, respectively, were returned. The median age of the students was 11 years (range 5–13 years).

Of the 233 responses, 12 (5%) were from households with case-patients in the initial cluster of pandemic (H1N1) 2009 infections that led to the recommendation for school closure; 143 (61%) of the responses were from households with contacts of case-patients, and 78 (34%) were from households with peers (Figure 2).

Of 221 contacts and peers, 19 (9%) reported onset of respiratory symptoms during the week of school closure; 14 were contacts and 5 were peers. Illness in 6 of the symptomatic students (3 contacts and 3 peers) met the criteria for ILI; the remaining illnesses were URIs (Table 1).

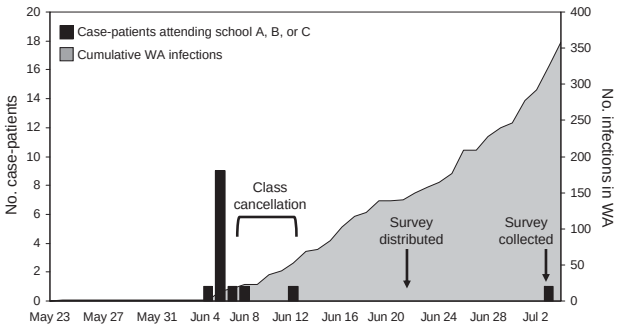

Figure 1. Confirmed pandemic (H1N1) 2009 influenza infections in Western Australia (WA), by onset date, May 23–July 4, 2009.

A total of 172 students (74%) reported going outside the home during the school closure period. In aggregate, students reported a total of 860 out-of-home activities during the school closure, resulting in a mean of 3.7 activities per student/week (a mean of 2.2 out-of-home activities per student during the 5 weekdays and 1.5 on the weekend). The number of out-of-home activities reported by individual students ranged from 0 to 24 (median 3 activities). Common reasons for going outside the home included sporting events, outdoor recreation, shopping, and parties (Table 2).

There was a significant difference in the proportion of case-patients (42%), contacts (66%), and peers (92%) who reported going out of the home ≥1 time during the closure period (Pearson $\chi^2 = 24.4$, df = 2, $p<0.0001$). The mean number of times students reported going out of the home was also associated with whether the student was a case-patient, contact, or peer; case-patients reported an average of 0.8 out-of-home activities per student per week compared with contacts and peers, who reported a mean of 2.9 and 5.6 activities per student per week, respectively (Kruskal-Wallis H = 35.1, df = 2, $p<0.0001$).

A total of 91 (45%) parents of 202 asymptomatic students reported taking ≥1 day (range 1–5 days; median 3 days) off work to care for their child during the closure period. Seventy-one parents (35%) of asymptomatic students reported having to make special childcare arrangements as a result of the school closures. The median number of days that special childcare arrangements were required was 2 (range 1–5 days). Twenty students (10%) cared for themselves at home for at least a portion of the closure period. Of the 202 asymptomatic students and of 31 ill students, 38 (19%) and 2 (6%), respectively, were cared for in a setting with children other than their siblings.

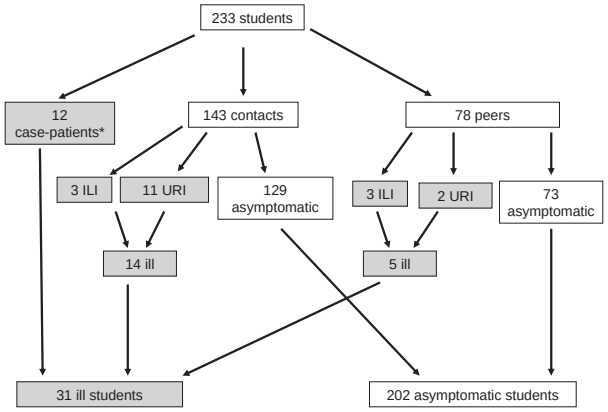

Figure 2. Distribution of student respondents by case or exposure classification and history of illness during a pandemic (H1N1) 2009–related school closure, Perth, Western Australia, June 8–14, 2009. URI, upper respiratory infection; ILI, influenza-like illness.

Of the 233 parents who returned the survey, 110 (47%) thought the school closure was appropriate, 76 (33%) thought it was inappropriate, and 47 (20%) were unsure. The percentage of parents who indicated that the closure was appropriate was highest among the parents of case-patients (92%), followed by the parents of contacts (48%), and peers (39%). Of those parents who thought school closure was appropriate, the main reason cited was to "protect the community"; of parents who thought the school closures were not appropriate, the main reason cited was "swine flu illness is mild" (Table 3).

Parental opinion about the appropriateness of the school closure was significantly correlated with student participation in activities outside the home. Students of parents who thought the school closure was not appropriate reported a mean of 4.7 out-of-home activities, compared with a mean of 4.3 activities for students of parents who were unsure and 2.8 for students of parents who thought the closure was appropriate (Kruskal-Wallis H = 14.9, df = 2, p = 0.0006). This pattern persisted when the analysis was restricted to the 202 students who were asymptomatic (Kruskal-Wallis H = 7.1, df = 2, p = 0.03).

Ninety percent of parents reported that the school closure caused minimal or no anxiety for their child, but 55% reported that school closure caused moderate or severe disruption to family routines. Forty-five percent indicated that they were well prepared for school closure (Table 3).

Discussion

This report describes the activities of students affected by school closure in response to the outbreak of pandemic (H1N1) 2009 and the effect of the closures on families. It is important to place these findings in context to fully understand their importance. The school closures in Perth occurred at a time when experience with pandemic (H1N1) 2009 was limited but transmission of the virus was accelerating across Australia. By June 8, 2009, >1,600 confirmed cases of pandemic (H1N1) 2009 illness had been reported nationally, and 10 fatal infections had received extensive media coverage. Thermal scanners were operational at Australia's international airports, and cruise ships with suspected case-patients had recently been quarantined at sea (*5*,*6*). During this phase of the response, case-patients and contacts were routinely provided with oseltamivir and instructed to voluntarily isolate or quarantine themselves at home. In addition, as a precaution, all school-aged children who traveled to areas with high rates of pandemic (H1N1) 2009, specifically the United States, Canada, Mexico, and Victoria, Australia, were being asked to remain in home quarantine for 7 days after return (*7*,*8*). The school closures in Western Australia were covered widely by the local media, and the message conveyed was that the school children were to be placed under "home quarantine" (*9*–*11*).

Table 1. Symptoms reported by contacts and peers who became ill during pandemic (H1N1) 2009–related school closures, Perth, Western Australia, Australia, June 8–14, 2009

Sign or symptom	No. (%) persons with ILI,* n = 6	No. (%) persons with URI,† n = 13	Total no. (%) persons, n = 19
Fever	6 (100)	1 (8)	7 (37)
Sore throat	6 (100)	6 (46)	12 (63)
Headache	5 (83)	4 (31)	9 (47)
Cough	4 (67)	4 (31)	8 (42)
Runny nose	4 (67)	7 (54)	11 (58)
Sneezing	3 (50)	2 (15)	5 (26)
Chills	2 (33)	0	2 (11)
Fatigue	1 (17)	1 (8)	2 (11)
Muscle aches	0	2 (15)	2 (11)
Diarrhea	0	1 (8)	1 (5)
Vomiting	0	2 (15)	2 (11)
Other	0	4 (31)	4 (21)

*ILI, influenza-like illness; defined as a history of fever with cough, sore throat, or both.
†URI, upper respiratory infection; defined as an illness not meeting ILI criteria but with ≥1 of the following: sore throat, cough, or runny nose.

Table 2. Number and type of out-of-home activities reported by 233 students during pandemic (H1N1) 2009–related school closures, Perth, Western Australia, Australia, June 8–14, 2009

Activity type	No. students reporting activity		
	Monday–Friday (Jun 8–12)	Saturday–Sunday (Jun 13–14)	Total
Going to a sporting event	86	128	214
Going to a park or beach	144	52	196
Going to a grocery store	68	35	103
Going to a shopping mall	63	28	91
Participating in an unspecified event or activity	58	17	75
Attending a party	28	37	65
Attending a music or art lesson	19	8	27
Going to a restaurant	13	14	27
Going to a movie	16	5	21
Attending a religious service	1	16	17
Attending a sleepover	10	6	16
Going to a tutoring lesson	6	2	8
Total	512	348	860

In this setting, less than half of parents (47%) felt that closing schools was an appropriate response to identification of pandemic influenza cases among students. Most parents who felt the closure was inappropriate indicated that their opinion was based on impressions about the disease itself (i.e., that it was generally mild and/or that transmission was not likely to be stopped by closing schools). Personal inconvenience, such as lost work or wages and childcare issues, seemed to be less important.

The effect of the closure on families was substantial. Almost half of parents reported missing work because of the school closure, and a third had to make special childcare arrangements. These special arrangements resulted in >1 of 6 students being cared for with children other than their siblings.

During the school closure, respiratory illness developed in 14 (10%) of 143 contacts and 5 (6%) of 78 peers; 6 of the 19 illnesses met the case definition for ILI, but the remaining URIs were mostly afebrile. Anecdotal reports from Mexico and elsewhere suggest that 30% of illnesses caused by pandemic (H1N1) 2009 may lack fever, and volunteer challenge studies with other influenza subtype H1N1 viruses have found the proportion of afebrile infections to be even higher (63%) (*12*,*13*). Clinical specimens were not available from ill students reporting ILI or URI in this assessment, so we are not able to establish the true incidence of influenza in our cohort.

Students commonly reported participating in activities outside the home during the school closure. Almost three quarters of all students left home at least 1 time during the 7-day reporting period.

This survey provides quantitative data on the frequency of students' out-of-home activities during school closures undertaken to control an outbreak. Understanding what children do when schools are closed is crucial to predicting the health effects of illness-related school closures (*14*,*15*). The 233 students in our survey reported, in aggregate, >850 out-of-home activities over 7 days. This may actually be an underestimate as it represents only what was later recalled by parents (*16*).

Studies attempting to simulate the impact of nonpharmaceutical interventions on pandemic transmission have found that the anticipated benefits of school closures may be substantially undermined if children are not sufficiently isolated (*14*,*17*). Simulation models that include school closures are sensitive to assumptions regarding the level of nonschool contacts that occur (*18*,*19*). The large potential reductions in disease transmission predicted by some modeling studies are often based on the assumption that students stay at home during school closures or that compliance with social distancing recommendations is high (e.g., 90%) (*20*,*21*). Our data suggest the assumption that children will be kept at home may not be realistic for the current outbreak of pandemic (H1N1) 2009. Of the 3 student cohorts we surveyed (case-patients, contacts, and peers), peers are likely to be representative of the majority of children affected during a large-scale school closure scenario; we observed that peers went out of the home nearly once a day, on average, during the survey period. How this behavior might have changed if the exclusion period had been longer is unknown.

It is likely that there is wide variability in terms of the risk for influenza transmission across the spectrum of outside-the-home activities the students engaged in (i.e., differences in duration and proximity of exposure to other persons). In our setting, sporting events (team practices and games) were the most common activity reported and have been recognized for their potential association with the spread of influenza (*3*). In contrast, excursions to a beach or park were also common, but these activities are probably less likely to result in disease spread. Parties were less frequent but not uncommon, and social events of this type have recently been implicated in transmission of pandemic (H1N1) 2009 in Europe (*22*). More work is needed to

Table 3. Household responses to survey regarding pandemic (H1N1) 2009–related school closures, Perth, Western Australia, 2009, June 8–14, 2009

Survey question, response	No. (%) responses
Was school closure appropriate, and why or why not?*	
Yes	110 (47)
To protect the community	84 (76)
To protect other students at the school	72 (66)
To protect my child and family	68 (62)
Swine flu illness is serious	31 (28)
No	76 (33)
Swine flu illness is mild	54 (71)
Cannot stop flu spread	50 (66)
Lost income due to missed work	18 (24)
Too difficult to make childcare arrangements	14 (18)
Not sure	47 (20)
What was the level of disruption to family routines caused by the closure?	
Severe	32 (14)
Moderate	95 (41)
Minimal	87 (37)
None	19 (8)
What level of anxiety did the closure create in your child?	
Severe	3 (1)
Moderate	20 (9)
Minimal	101 (43)
None	109 (47)
What could have helped you be bettered prepared for the school closure?*	
Nothing, we were well prepared	104 (45)
More time between notification and closing	47 (20)
Assistance with emergency childcare arrangements	45 (19)
Better understanding of potential length of closure at outset	26 (11)

*Reasons for the individual responses were not mutually exclusive.

clarify the likelihood of influenza transmission associated with different activities so recommendations on restricting students' public contact during school closures might be targeted at those interactions that pose substantial risk.

Another key finding of this assessment is that parental opinions about the appropriateness of school closure were correlated with whether the student participated in activities outside the home. If high rates of compliance with student-centered, nonpharmaceutical interventions are to be obtained, public health officials must communicate to parents why the intervention is warranted and explain the anticipated benefits to the community. It is critical, however, that health officials do "not overstate the level of confidence or certainty in the effectiveness of these measures" (*18*).

It seems reasonable to assume that behaviors reported by students during school closures would be highly influenced by the socioeconomic status of the population studied. We note, however, that our results parallel findings from school closures in rural North Carolina, USA, that were implemented in response to a seasonal influenza outbreak. Despite recommendations to avoid large gatherings, most (89%) of the North Carolina students visited at least 1 public location during the 10-day closure (*17*). The general consistency observed in terms of participation in ≥1 out-of-home activities across the North Carolina and Western Australia settings is noteworthy because the 2 communities seem to be quite distinct with regard to socioeconomic status. In our Western Australia study, the students attended a mix of private and public schools in a relatively urbanized, affluent community; in the North Carolina study, 41% of the participating households received free or reduced-cost lunches through a national school lunch program (an indicator of lower socioeconomic status). Marked differences between the Western Australia and North Carolina assessments were noted, however; for example, in North Carolina, >91% of households considered the school closures appropriate, and no adults reported missing work as a result of the closures (*17*). Additional studies among a wide array of communities will be needed to better elucidate the relationship between socioeconomic status and the effect of school closures on households and student behavior.

This evaluation was performed as a field assessment of an interim, evolving public health response and has several major limitations. First, data were obtained by using a retrospective self-administered questionnaire. Although the response rate (58%) was acceptable, the accuracy of recall may have declined between the end of the closure and the time that the questionnaire was distributed.

Second, we did not collect data on students who were not subject to school closures, so we cannot determine the

extent to which student participation in activities outside the home reported by our cohort may have been influenced by the school closure itself (i.e., compensatory behaviors). It is also possible that the number of out-of-home activities per student represents a substantial reduction from "normal" behavior, and thus the school closures could be considered a "qualified success" in terms of reducing student contacts. In addition, it is likely that the overall number of contacts among children and the density of children during the school day exceed the number and density that occur outside the school during a school closure. Whether these plausible, but as-yet-unquantified, relative reductions in student contacts would be sufficient to substantially reduce influenza transmission during a pandemic remains undetermined.

Third, we caution that the responses obtained from Western Australia may not be generalizable to other communities and cultures or to another pandemic with a different disease severity profile. In addition, our findings regarding out-of-school activities may not be applicable to situations in which a total school closure is accompanied by cancellation of all extracurricular school activities. In this assessment, school A closed entirely but schools B and C underwent only partial closure; because schools B and C closed only specific grades, their extracurricular activities were not cancelled and remained open to children in nonaffected grades.

Fourth, our questionnaire did not directly assess parents' knowledge about why classes were cancelled and what they understood about limiting their child's activities outside the home. Without this information, it is not possible to determine whether the degree of participation in out-of-home behavior was due to noncompliance or lack of communication.

On the basis of our experience, there are several recommendations future investigators may wish to consider when attempting to assess the effect of illness-related school closures conducted as a disease control measure. These recommendations include 1) directly assessing parental understanding of the recommendations to limit student activities outside the home, 2) concurrently determining the level of out-of-home activities for students at comparable schools not subject to closure, 3) specifically asking about healthcare-seeking behaviors during the school closure, and 4) inquiring about the extent to which any reported outside-of-home behaviors were undertaken as school-affiliated extracurricular activities.

In summary, this study contributes to the growing body of knowledge on student behavior during school closures and the effect of such closures on households. The results of our assessment may be helpful to public health and education officials considering school closure as a means to control an influenza outbreak. In addition, these data might help inform assumptions underpinning studies that estimate the effect of nonpharmaceutical interventions. The general paucity of data on student activities during school closures, however, remains a major barrier to understanding the potential effect of closures as a disease mitigation measure and underscores the need for further research (*18*,*23*–*25*).

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Employment and Compliance with Pandemic Influenza Mitigation Recommendations

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In the event of a serious pandemic influenza outbreak, businesses must play a key role in protecting employees' health and safety. With regard to pandemic influenza mitigation recommendations requiring social distancing, we examined whether some US employees would disproportionately fail to comply because of job insecurity and financial problems associated with missing work. We used the 2006 Harvard School of Public Health Pandemic Influenza Survey and multivariable logistic regression to determine whether employment characteristics such as inability to work from home, lack of pay when absent from work, and self-employment would be associated with less ability to comply with recommendations. We found that inability to work from home, lack of paid sick leave, and income are associated with working adults' ability to comply and should be major targets for workplace interventions in the event of a serious outbreak.

The world needs a detailed operational blueprint for the best way to get through 12–24 months of a pandemic influenza outbreak; that type of planning must be on the agenda of every public health agency, school board, state legislature, and business (*1*). In January 2008, the Centers for Disease Control and Prevention outlined several recommendations (*2*). In the event of a serious outbreak, employers must play a key role in protecting employees' health and safety (*2*). Specifically, businesses should "forecast and allow for employee absences during a pandemic due to factors such as personal illness, family member illness, community containment measures and quarantines, school and/or business closures, and public transportation closures" and workers should "plan for the possible reduction or loss of income if [they are] unable to work or if place of employment is closed" (*2*).

Should social distancing from the workplace become a reality, some members of the US workforce may be disproportionately vulnerable to compliance failure and negative outcomes of an influenza pandemic because of real and perceived job insecurity and financial problems associated with missing work. Previous research suggests that compliance with recommendations in emergency situations reflects the interaction of many modifiable and nonmodifiable factors, including how persons perceive their personal and family risk, what resources they have available, what negative consequences they anticipate as a result of compliance, their socioeconomic status, and how well official planning efforts are organized (*3*–*6*). Attitudes toward the use of social distancing to mitigate outbreaks of severe acute respiratory syndrome, smallpox, or avian influenza may be influenced by largely modifiable problems that people associate with isolation, such as not being able to get healthcare or prescription drugs and losing pay or jobs for missing work (*7*). Indeed, more than one third of US employees say that they would not get paid if they had to stay home from work because of a severe outbreak of pandemic influenza, and less than one third believe that they could work from home for 1 month (*8*).

We hypothesized that working adults who are unable to work from home and who do not have sick leave will have less ability to comply with pandemic influenza isolation recommendations that require missing work because of fear of losing their job or business and serious financial problems that would arise from missing work. To test our hypothesis, we assessed the relative independent contribu-

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tion of selected employment and sociodemographic characteristics on working adults' ability to comply with pandemic influenza mitigation strategies involving workplace isolation.

Methods

Data

We used data from the 2006 Harvard School of Public Health (HSPH) Pandemic Influenza Survey, a random digit–dial survey sponsored by the HSPH Project on the Public and Biological Security. The survey was conducted to provide information with regard to the public's reaction to the possible use of social distancing and other nonpharmaceutical interventions during a severe outbreak of pandemic influenza. Survey questions assessed willingness and ability to comply with public health recommendations in 3 domains: home, school, and work.

The target population was adults ≥18 years of age, who lived in the United States. During September 28 through October 25, 2006, International Communications Research (Media, PA, USA) surveyed a representative sample of adults ≥18 years of age, including an oversample of adults with children <18 years of age in the household. Sampling procedures are described elsewhere (*8*). Response rate was 36% and cooperation rate was 75%, which produced a total of 1,697 completed interviews (*8*). Because adults with children were oversampled to gauge the possible effect of community mitigation on families, data were weighted to reflect the actual proportion of the total adult population. In addition, to compensate for nonresponse bias and unequal probability of selection and to ensure that demographic groups were represented in their actual proportion in the adult population, we weighted sample data to the most recent US census data available from the Current Population Survey (www.census.gov/cps) for gender, age, race, region, and education.

Employment characteristics for the full sample were 50% employed full time, 13% employed part time, 36% unemployed, and 1% unknown. Our study focused on employment-related constraints that may limit ability to comply with isolation recommendations; therefore, we analyzed responses from only the 1,101 respondents who were employed either full or part time (Table 1). A small percentage of employed respondents refused to answer some items in the survey (e.g., 15% refused income, 3% education, 2% race).

Table 1. Sample characteristics of 1,101 employed respondents, 2006 Harvard School of Public Health Pandemic Influenza Survey*

Characteristic	% Respondents
Female	54
Age, y	
18–30	28
31–50	48
≥51	24
Annual household Income	
<$30,000	18
$30,000–$49,000	19
$50,000–$74,000	19
≥$75,000	29
Education	
Less than HS	12
HS graduate or HS plus technical school	29
Some college, no degree	25
College degree or more	31
Race/ethnicity	
African-American	11
Hispanic	14
Other	7
White	66
Residence	
Urban	73
Rural	27
Employment	
Part time	21
Full time	79

*HS, high school. Alll samples are weighted. Entries may not total 100% because of refused or missing responses.

Outcome Variables: Indicators of Ability to Comply

Employed respondents were asked a series of questions to assess real or perceived constraints with regard to their ability to comply with pandemic influenza mitigation strategies that require isolation from the workplace. The 4 dependent variables were positive responses to the following statements: "It is likely that I or a member of my household would lose a job or business as a result of having to stay home for 7–10 days" (dichotomized; "very" and "somewhat" likely represented a positive response and "not too" and "not at all" likely represented a negative response); and "I would have serious financial problems if I stayed away from work for 7–10 days, 1 month, or 3 months." The latter questions used a split sample, whereby only respondents who answered "no" or "don't know" to the 7–10 day duration were asked about financial problems that they would have at 1 month, and only those who answered "no" or "don't know" to having serious financial problems at 1 month were asked about the 3-month period. Many unadjusted prevalence estimates for each outcome variable (Table 2) have been described in a descriptive study that used the 2006 HSPH Pandemic Influenza Survey (*8*).

Predictor Variables

To assess the effect of potentially modifiable employment-related constraints on compliance with recommendations that require missing work, we chose key predictor variables that represented selected employment characteristics; i.e., inability to work from home, lack of pay when

Table 2. Prevalence estimates for responses of 1,101 employed respondents (unadjusted), 2006 Harvard School of Public Health Pandemic Influenza Survey*

Variable	No. responses	% Yes	% No	% Don't know
Outcome variables representing ability to comply with pandemic influenza mitigation recommendations				
If you were asked to stay home for 7–10 days and avoid contact with anyone outside your household, would you or someone in your household lose your job or business?	1,073	28	71	1
Would it become a serious financial problem if you stayed out of work for 7–10 days?	1,072	25	74	1
Would it become a serious financial problem if you stayed out of work for 1 month?	806†	42	55	2
Would it become a serious financial problem if you stayed out of work for 3 months?	464‡	45	51	4
Key predictor variables representing employment-related constraints on compliance				
Unable to work from home for 1 month in the event of a serious outbreak	1,073	69	29	2
Would not be paid if kept from work because of a serious outbreak	1,071	42	35	22
Self-employed	1,072	16	84	–

*All estimates are weighted. Cell counts may not total 100% due to refused or missing responses. Sample size for each question varies due to refused and missing responses.
†Split sample; question asked only of those who responded "no" or "don't know" to financial problems after 7–10 days.
‡Split sample; question asked only of those who responded "no" or "don't know" to financial problems after 1 month.

absent from work, and self-employment status (Table 2). With regard to employment-related constraints, <1% of respondents refused to answer 2 questions.

Covariates

To assess the potentially disproportionate difficulties that low-income and urban populations may face if asked to stay home from work in the event of a serious outbreak, we included income and urban residence in all models. Other sociodemographic and personal characteristics assessed were education, race/ethnicity, age, sex, self-reported health status, and self-reported knowledge of pandemic influenza.

Statistical Analysis

Structured analytical approaches have improved predictions of behavior in emergency situations, modeling the joint effects of several factors on planned behavior (*3*). Therefore, to identify factors that may lead to disproportionate vulnerability in the event of a serious outbreak, we used multivariable logistic regression to model the predicted probability that some groups of working adults (delineated by employment characteristics such as inability to work from home, lack of pay when absent from work, and self-employment) may be less able than identified referent groups to comply with pandemic influenza mitigation strategies that require voluntary isolation from work.

Each outcome used 1 full model; all models controlled simultaneously for all key predictors as well as covariates such as income, urban residence, age, race/ethnicity, education, gender, self-reported health status, and self-reported knowledge of pandemic influenza. We conducted a complete case analysis and analyzed only those 1,101 respondents who reported being employed either full or part time. Tests of significance were estimated at $p<0.05$, and 95% confidence intervals (CIs) were reported for all odds ratios (ORs). To adjust for unequal probabilities of selection and for potential nonresponse bias, we applied individual weighting factors to all estimates. The analysis was conducted with SAS software version 9.2 (Cary, NC, USA) by using the PROC SURVEYLOGISTIC procedure and probability sampling weights.

Results

Perceived Likelihood of Losing Job or Business

Of the employed respondents, 28% reported that they likely would lose their job or business as a result of having to stay home from work for 7–10 days in the event of a pandemic influenza outbreak (Table 2). Our multivariable models elucidated differential vulnerability by lack of paid sick leave, income level of respondents, and urban residence (Table 3). Those respondents who would not be paid if kept from work were almost 5× more likely than those who would receive pay (OR 4.72) to say that they would likely lose their job or business as a result of having to stay home from work for 7–10 days.

Respondent income also was associated with reported likelihood of losing a job or business. Those who earned <$30,000 per year were 4× more likely than those who earned ≥$75,000 per year (OR 4.31) and those who earned $30,000–$49,000 per year and $50,000–$74,000 per year were ≈2× more likely than those who earned ≥$75,000 per year (ORs 1.70 and 2.08, respectively) to say that they would likely lose their job or business as a result of having to miss work in the event of a serious outbreak.

Urban residence was associated with limited ability to comply with recommendations that require missing work. Respondents living in urban areas were ≈70% more likely than those living in rural areas to say that they would likely lose their job or business as a result of having to stay home for 7–10 days in the event of an outbreak (OR 1.66).

Perceived Likelihood of Experiencing Serious Financial Problems

Certain employment characteristics and respondent income levels were associated with the likelihood that working adults would experience serious financial problems and thus be less able to comply with isolation recommendations, if required to miss work for long periods of time (Table 3). Respondents who were self-employed were twice as likely as those who worked for an employer to say that they would experience serious financial difficulties if isolated from work for 7–10 days (OR 2.09). Those who were not able to work from home were significantly more likely than those who were able to work from home to say that they would experience serious financial problems if isolated from work for durations of 7–10 days, 1 month, and 3 months (ORs 1.57, 1.91, 1.65, respectively).

Respondent income also was associated with likelihood of experiencing serious financial problems if the respondent were kept from work because of an outbreak of pandemic influenza, although the models showed interesting patterns, depending on the duration of isolation. If isolated from work for 7–10 days, those who earned <$30,000 per year were 3× more likely than those who earned ≥$75,000 per year to say that they would experience substantial financial problems (OR 3.26). At 1 month of isolation, all those in low- and middle-income groups

Table 3. Likelihood of compliance with work-related pandemic influenza isolation strategies, by employment-related constraints and sociodemographics*

Variable	Referent	Likelihood,† OR (95% CI)			
		Lose job or business, n = 928‡	Serious financial problems		
			7–10 d, n = 927§	1 mo, n = 754¶	3 mo, n = 472#
Employment-related constraints					
Unable to work from home for 1 mo	Able	0.99 (0.63–1.56)	**1.57 (1.02–2.51)****	**1.91 (1.30–2.79)††**	**1.65 (1.06–2.52)****
Would not be paid if kept from work	Paid	**4.72 (2.94–7.57)††**	**3.23 (2.03–5.13)††**	**2.93 (2.07–4.14)††**	**1.75 (1.14–2.62)‡‡**
Self-employed	Works for someone else	1.09 (0.64–1.85)	**2.09 (1.25–3.49)‡‡**	0.68 (0.42–1.14)	0.66 (0.38–1.16)
Sociodemographic characteristics					
Income	≥$75,000				
<$30,000		**4.31 (2.43–7.63)††**	**3.26 (1.85–5.75)††**	**3.29 (1.78–6.05)††**	**3.52 (1.38–8.98)‡‡**
$30,000–$49,000		**1.70 (1.01–3.02)****	1.57 (0.93–2.64)	**2.93 (1.81–4.75)††**	1.46 (0.79–2.69)
$50,000–$74,000		**2.08 (1.25–3.48)‡‡**	1.09 (0.64–1.84)	**1.89 (1.25–2.88)‡‡**	1.27 (0.78–2.07)
Urban residence	Rural	**1.66 (1.07–2.56)****	1.30 (0.84–2.01)	1.14 (0.77–1.68)	0.66 (0.41–1.06)
Education	College				
Less than HS		2.40 (0.84–6.80)	1.73 (0.62–4.80)	1.24 (0.36–4.28)	0.45 (0.06–3.29)
HS graduate or HS plus technical school		**2.03 (1.17–3.51)‡‡**	1.62 (0.95–2.75)	1.17 (0.69–1.99)	0.41 (0.20–1.07)
Some college		1.15 (0.63–2.05)	1.25 (0.73–2.14)	0.89 (0.55–1.42)	0.71 (0.38–1.31)
Race/Ethnicity	White				
African-American		1.74 (0.92–3.29)	0.56 (0.26–1.18)	1.51 (0.77–2.95)	0.73 (0.31–1.70)
Hispanic		1.55 (0.83–2.88)	0.65 (0.32–1.34)	0.74 (0.37–1.48)	1.52 (0.65–3.57)
Other		2.23 (0.92–5.43)	1.22 (0.45–3.26)	0.71 (0.27–1.86)	0.81 (0.29–2.31)
Age, y	≥51				
18–30		**1.99 (1.09–3.66)****	1.08 (0.56–2.05)	0.73 (0.40–1.32)	1.54 (0.75–3.15)
31–50		1.09 (0.71–1.71)	1.49 (0.96–2.33)	1.04 (0.71–1.53)	1.38 (0.88–2.16)
Gender	F	0.77 (0.53–1.13)	0.84 (0.57–1.22)	1.07 (0.76–1.51)	1.07 (0.72–1.61)
Good health status	Poor health	0.58 (0.29–1.13)	**0.50 (0.25–0.97)****	0.96 (0.40–2.29)	0.60 (0.22–1.69)
Knowledge of pandemic influenza	Never heard of	0.59 (0.37–0.96)	0.79 (0.49–1.30)	1.23 (0.73–2.06)	1.02 (0.53–1.94)
–2LL		735.72	740.38	860.05	605.98

*Multivariable fitted logistic regression models describing the odds that some groups may be less able than identified referent groups to comply with pandemic influenza mitigation strategies that require voluntary isolation from work. All estimates are weighted and control for age, race/ethnicity, education, gender, self-reported health status, and self-reported knowledge of pandemic influenza. OR, odds ratio; CI, confidence interval; HS, high school; LL, log likelihood. **Boldface** indicates significance at p<0.05.
†Response of persons employed full or part time to "If pandemic influenza remained in your community for some time, health officials might recommend that people stay home from work so they do not catch or spread the disease."
‡I or a member of my household would lose job or business as a result of having to stay home for 7–10 days.
§I or a member of my household would have serious financial problems if I stayed away from work for the following period of time.
¶Split sample; question asked only of those who responded "no" or "don't know" to financial problems after 7–10 days.
#Split sample; question asked only of those who responded "no" or "don't know" to financial problems after 1 month.
**p<0.05.
††p<0.0001.
‡‡p<0.01.

were significantly more likely than those in the highest income group to say that they would experience serious financial problems if kept from work. There was a fine income gradient; those earning <$30,000 per year were 3.29× more likely, those earning $30,000–$49,000 per year were 2.93× more likely, and those earning $50,000–$74,000 per year were 1.89× more likely than those earning ≥$75,000 per year to say that staying away from work for 1 month would pose serious financial problems. At 3 months of isolation, the trend shifted somewhat. Low-income workers were still significantly more likely than high-income workers to say that they would have serious financial problems if isolated from work (OR 3.52), indicating disproportionate vulnerability for low-income populations across all durations of isolation. However, at 3 months, middle-income workers would not be more or less likely than those earning ≥$75,000 to say that they would experience serious financial problems, indicating that a 3-month period of isolation would likely be difficult for those in all income groups, including those in the highest income categories.

Discussion

The threat of a human influenza pandemic has greatly increased over the past several years with the emergence of highly virulent avian influenza viruses, notably subtype H5N1 (*9*), and the more recent emergence of subtype H1N1. Federal agencies have modeled the high probability of a serious pandemic influenza outbreak and have begun to institute national and state plans to reduce transmission and mitigate the disease (*10*). The inadequate supply of some vaccines and antiviral medications and insufficient community mitigation planning have led to concern that the United States is inadequately prepared to deal with a pandemic (*11*).

Improving pandemic preparedness is critical, given the catastrophic consequences of influenza pandemics that have occurred in the past century, in 1918, 1957, and 1968; the severity of all past pandemics was substantial, ranging from 700,000 deaths (in 1968) to >50 million deaths (in 1918) (*1*,*11*). Evidence to determine the most effective nonpharmaceutical intervention strategies is limited (*12*). Some strategies being suggested include targeted, layered containment (*13*), which involves antiviral drug treatment for identified case-patients and prophylaxis for and quarantine of their household members, school closures, and social distancing in the community and workplace (*2*,*8*,*13*,*14*). Research has suggested that US adults seem to possess a broad willingness to comply with response strategies that include social distancing, although some segments of the population will likely be less able to comply with isolation recommendations (*8*), particularly those related to isolation from the workplace.

This study may provide public health authorities with realistic expectations for the success or failure of proposed mitigation measures, given that some population subgroups may have less ability to comply with recommendations because of real or perceived job insecurity and financial problems associated with missing work. Our findings suggest that some employment characteristics (inability to work from home, lack of paid sick leave) are associated with working adults' ability to comply with recommendations and will be major workplace intervention points (areas to target) in the event of a serious outbreak. In addition, sociodemographic characteristics (particularly low-income status) put some workers at disproportionate risk of contracting and spreading pandemic influenza because of their perceived inability to miss work. These assessments may help identify the conditions under which some groups will be disproportionately likely to fail to comply and may help with workplace efforts to plan accordingly and communicate effectively in the event of a serious outbreak of pandemic influenza.

Job insecurity, whether real or perceived, is a real consideration for many working adults. US health authorities recommend that to prepare for a pandemic, businesses should establish policies for nonpunitive liberal leave and flexible worksite accommodations (*2*). However, we know of no legal precedent for mandatory job protection in the event of public health emergencies. Our study found that employees without paid sick leave, those with low income, and those who live in urban areas fear losing their jobs should they comply with recommendations to stay home in the event of a serious outbreak of pandemic influenza. Those respondents who said that they would not be paid if kept from work were almost 5× more likely as those who would receive pay to say that they would lose their job or business as a result of having to stay home from work. We were not surprised by this finding, given the long history of social epidemiology literature (e.g., the Whitehall studies) that has documented the effect of occupational status or grade, organizational injustice, job stress, and workplace power differentials on both job insecurity and disease outcomes (*15*–*20*). The effect of lack of paid sick leave provides insight into a measure of inequality in the work force, such that some groups of employees (e.g., those in minimum wage jobs or without paid sick leave), because of concerns about job security that stem from their workplace status, lack the power to choose to stay home from work in the event of an outbreak. Notably, across all income categories, low- and middle-income workers were significantly more likely than high-income workers to say that they would be likely to lose their job or business as a result of staying home for 7–10 days in the event of an outbreak. Those respondents living in urban areas also were 60% more likely than those living in rural areas to fear job insecurity. This

fear could pose substantial problems for pandemic influenza mitigation because those in urban areas may be strongly encouraged to remain isolated to avoid virus spread in conditions of population density and crowding.

Financial problems also are likely to weigh heavily on the minds of US workers during a pandemic, and these problems may be part of the complexity of factors that comprise compliance considerations. Although US health authorities have recommended that businesses develop policies for employee compensation in the event of an influenza pandemic that causes workplace absences (*2*), we know of no precedent requiring that paid sick leave be granted (by employers or state or federal government) to employees who comply with isolation recommendations and miss work in the event of a public health emergency. Our study has elucidated some employment characteristics that are associated with the likelihood that workers think they would experience serious financial problems if they had to miss work; inability to work from home and lack of paid sick leave were associated with reports of experiencing serious financial problems if isolated from work over the 3 periods: 7–10 days, 1 month, and 3 months. Respondent income was another significant predictor of serious financial problems that may limit ability to comply with isolation recommendations. Even relatively short periods of isolation from the workplace (7–10 days) would be a problem for low-income workers, and if an outbreak were serious enough to warrant 1-month isolation recommendations, persons in low- and middle-income groups would have more difficulty complying than would upper-income groups, thus limiting the effectiveness of mitigation strategies. Moreover, at 3 months' of isolation, persons from all income levels, especially low-income, would likely experience serious financial problems.

Strengths of our study include its practical significance; our findings may help preparedness planners find work-specific strategies that may increase the likelihood of compliance with isolation recommendations. These strategies may include working with employers to ensure work-from-home or sick leave capabilities for nonessential employees and planning to provide state or federal supplementary income support and job protection for workers who would not be paid if they missed work because of official pandemic mitigation recommendations. Other countries have implemented similar measures in emergencies; for example, during the 2003 pandemic of severe acute respiratory disease, the government of Singapore provided financial support to citizens who had to stay home to prevent the spread of the disease (*21*). In the United States, no such measures have been taken to prevent the spread of infectious diseases, but for other emergencies such as hurricanes and floods, federal income support has been provided to victims by way of disaster relief funds. In the event of a serious outbreak of pandemic influenza, when timely action will be needed to encourage and ensure isolation compliance, an existing mechanism for delivering financial support to affected persons is unemployment insurance. Currently, eligibility requirements for this benefit are limited to employees who involuntarily lose their job, but this requirement could be changed to use an existing system to disperse lump-sum payments to those financially affected by a pandemic, if the severity of an outbreak warranted isolation from the workplace for long periods.

Findings from our study should be considered in light of a few limitations, including the 36% response rate. Low response rates can bias samples, reflecting systematic differences between responders and the population from which they were drawn, thus limiting the external validity of estimates (extrapolation to the general population). However, the 1-month period of the survey (and thus limited time for callbacks) may mirror what might be necessary in the event of a pandemic, in which public surveys with a rapid turnaround time are necessary to gauge public knowledge and resource needs in an emergency situation. We point to research that suggests that the results of weighted data from surveys of shorter duration are similar to those based on surveys of longer duration and higher response rates and can be used without an unacceptable risk for bias (*22*,*23*). Furthermore, the HSPH Pandemic Influenza Survey, as it relates to our findings about job insecurity, did not assess perceptions of job loss versus reality of job loss, nor did it assess reasons why some respondents perceived that job loss would be a consequence for missing work for 7–10 days in the event of a serious outbreak. Future population surveys could attempt to disentangle these beliefs to inform policy and communication aimed at enabling compliance with workplace isolation strategies to quell the spread of a future pandemic.

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Human Hendra Virus Encephalitis Associated with Equine Outbreak, Australia, 2008

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A recent Hendra virus outbreak at a veterinary clinic in Brisbane, Queensland, Australia, involved 5 equine and 2 human infections. In contrast to previous outbreaks, infected horses had predominantly encephalitic, rather than respiratory, signs. After an incubation period of 9–16 days, influenza-like illnesses developed in the 2 persons before progressing to encephalitis; 1 died. Both patients were given ribavirin. Basal serum and cerebrospinal fluid levels were 10–13 mg/L after intravenous administration and 6 mg/L after oral administration (isolate 90% inhibitory concentration 64 mg/L). Both patients were exposed to infected horses, 1 during the late incubation period in a horse. The attack rate for veterinary clinic staff exposed to infected horses was 10%. An isolate from this outbreak showed genetic heterogeneity with isolates from a concurrent, but geographically remote, outbreak and from previous outbreaks. Emergence of Hendra virus is a serious medical, veterinary, and public health challenge.

The genus *Henipavirus* of the family *Paramyxoviridae* contains 2 recently described viruses, Hendra virus and Nipah virus, whose natural reservoir is fruit bats of the genus *Pteropus* (*1*). Hendra virus has caused serious respiratory infections in horses and respiratory and neurologic infections in humans. After 2 Hendra virus outbreaks in 1994, which involved 22 horses and 3 humans (*2*,*3*), a total of 7 additional equine infections and 1 human infection were documented up to 2008 (*4*,*5*); all occurred in coastal Queensland and northern New South Wales in Australia. Of the 4 persons with documented Hendra virus infection, 2 recovered from influenza-like illnesses (ILIs) (*2*,*5*) and 2 died, 1 from respiratory failure (*2*) and 1 from encephalitis 13 months after initial aseptic meningitis (*3*). The estimated incubation period in humans of 7–8 days was based on these cases.

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The Outbreak

In early July 2008, a veterinary practice (clinic) in Thornlands, Queensland, was quarantined after 2 acutely ill horses were provisionally diagnosed with Hendra virus infection. Four horses eventually died from the infection, and another was humanely killed after it recovered, in accordance with established national veterinary practice (*6*). In contrast to previous equine outbreaks, horses in this outbreak showed predominantly neurologic (ataxia, head tilt, limb paresis), rather than respiratory, symptoms (*1*). We report 2 additional human cases of Hendra virus encephalitis, 1 fatal, in veterinary workers associated with this equine outbreak.

Patient 1

A 33-year-old man (equine veterinarian) at the clinic had a 2-day history of an ILI (fever, myalgia, and headache) in mid-July 2008. Clinical examination showed only a fever (38°C); mild neutropenia (0.7×10^9 cells/L) and thrombocytopenia (79×10^9 cells/L); a normal chest radiograph; and negative PCR results for respiratory viruses, including influenza, on a nasopharyngeal aspirate (NPA) specimen. Hendra virus RNA was detected by reverse transcription–PCR (RT-PCR) in serum and NPA specimens. The patient remained clinically well and showed defervescence on day 4 of illness.

By day 5, mild confusion, ataxia, bilateral ptosis, but no other neurologic signs, developed. Magnetic resonance imaging (MRI) showed multifocal (bilateral, cerebral, cortical, right pontine, scattered white matter) hyperintense lesions on T2 flair images associated with evidence of infarction on diffusion weighted images. A cerebrospinal fluid (CSF) sample had a leukocyte count of 4×10^6 cells/L, a protein level of 600 mg/L, and a glucose level of 3.7 mmol/L; Hendra virus RNA was detected by RT-PCR. An electroencephalogram (EEG) showed bilateral high-voltage slow waves but no epileptiform activity. He was treated with intravenous ribavirin (30 mg/kg initial dose, then 15 mg/kg every 6 h for 4 days, and 8 mg/kg every 8 h thereafter); enteric aspirin (100 mg/day); and because of progressive confusion and ataxia, intravenous dexamethasone (4 mg every 6 h).

Over the next 4 days (to day 10) progressive neurologic signs and high-grade fever developed, culminating in a generalized partial tonic-clonic seizure. During the next 4 weeks, the patient required mechanical ventilation, remained febrile, and despite control of seizure activity and minimal sedation, remained deeply unconscious with minimally reactive pupils. Treatment with ribavirin was stopped after 12 days of therapy when the patient's hemoglobin level decreased to 76 g/L. Serial MRI showed marked progression with innumerable cortical, subcortical, and brainstem hyperintense foci on T2 flair images with matching diffusion restriction, and leptomeningeal enhancement (Figure 1, panel A). Serial EEGs showed an absence of a stable background rhythm, slow wave activity with periodic sharp discharges, and a right-sided epileptogenic focus. CSF on day 28 had a leukocyte count of 84×10^6 cells/L, a protein level of 650 mg/L, and a glucose level of 4.3 mmol/L; Hendra virus RNA was not detected by RT-PCR. He died on day 40 of illness. A postmortem examination was not performed because a risk assessment conducted by Queensland Health concluded that an examination could not be performed in Queensland.

Patient 2

A 21-year-old woman (veterinary nurse) at the same clinic was observed 4 days after patient 1 with a 3-day history of an ILI. Results of clinical examination were unremarkable apart from a fever (39°C). Results of initial investigations, including complete blood examination, chest radiography, and EEG, were normal. Hendra virus RNA was detected in serum and NPA specimens. She was treated with intravenous ribavirin (30 mg/kg initial dose, then 15 mg/kg every 6 h) and aspirin (100 mg/day). On day 12 of illness, 96 hours after defervescence, encephalitic manifestations (mild confusion, dysarthria, ataxia, and bilateral ptosis) developed. Results of MRI and a CSF specimen were normal; Hendra virus was not detected by RT-PCR.

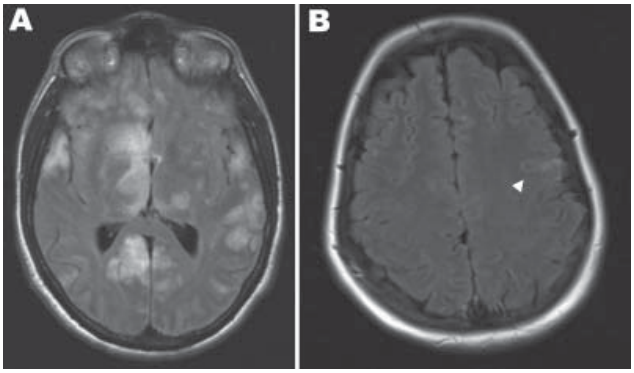

Figure 1. Magnetic resonance imaging scans of the brains of 2 patients with Hendra virus encephalitis, Australia, 2008. A) Patient 1 on day 18 of illness, showing cortical and subcortical hyperintense foci. B) Patient 2 on day 25 of illness, showing hyperintense foci in the left precentral gyrus (arrowhead).

Over the next 12 days, signs of encephalitis deteriorated (ataxia, dysarthria) before stabilizing. Serial MRI showed scattered, small, hyperintense, cortical and subcortical foci on T2 flair images and leptomeningeal enhancement (Figure 1, panel B), and EEGs showed severe diffuse encephalopathy with high-amplitude slow waves. Clinical and EEG improvement ensued, and the patient was discharged on day 37 with mild residual ataxia. By then, she had received 32 days of intravenous ribavirin and had steady-state basal serum ribavirin levels of 10.1 mg/L and 13.1 mg/L and a CSF level of 9.9 mg/L (all obtained while she was receiving 15 mg/kg every 6 h; J. Ray, pers. comm.).

Treatment with ribavirin was continued at a dosage of 600 mg orally every 8 h (basal level 6.2 mg/L; J. Ray, pers. comm.), and she remained clinically well during 20 weeks of follow-up. Repeat MRI (at days 69 and 135 and month 11) showed marked resolution in signal abnormalities, and EEGs (at days 69 and 182) also showed considerable improvement. A CSF sample obtained at month 12 showed normal results, including a negative result for Hendra virus by RT-PCR.

Results

Virologic and Serologic Findings

Serial quantitative RT-PCR for Hendra virus RNA (*7*) in serum, NPA, and urine specimens and serial immunofluorescent antibody assay and microsphere immunoassay for immunoglobulin (Ig) G and IgM against Hendra virus (*8*) in serum were performed for both patients (online Appendix Table, www.cdc.gov/EID/content/16/2/219-appT.htm). Seroconversion of IgG against Hendra virus occurred on days 5 and 11 in patients 1 and 2 respectively, and Hendra virus RNA was detected up to days 14 and 23, respectively.

Direct PCR sequencing of clinical material from both patients was used to obtain nucleotide sequences for the nucleoprotein, matrix, glycoprotein, and accessory protein genes and the intragenic junctions. Results showed that Hendra virus associated with this outbreak was distinct from Hendra virus in a concurrent equine outbreak in Cannonvale in northern Queensland, Australia, and from viruses in previous outbreaks (Figure 2).

Virus isolated from Vero E6 cells (CRL-1586; American Type Culture Collection, Manassas, VA, USA) cultured with an NPA sample obtained on day 5 of illness of patient 2 was used to test in vitro susceptibility to ribavirin (*9*). The 90% inhibitory concentration of ribavirin for this isolate was 64 mg/L.

Human Public Health Investigations and Responses

After confirmation of equine Hendra virus cases, 83 contacts of infected horses (37 veterinary clinic staff, 25 owners/handlers, and 21 Department of Primary Industries staff) and 8 contacts of human case-patients (6 domestic contacts, and 2 healthcare workers) were contacted for assessment and counseling. Nonspecific respiratory or gastrointestinal symptoms developed in 16 contacts of infected horses; none had Hendra virus RNA or antibodies against this virus in convalescent-phase serum samples (obtained ≥2 weeks after last exposure to infected horses or humans). One veterinary worker had percutaneous blood exposure while performing euthanasia on the Hendra virus–infected horse that recovered. This person was treated with a 5-day course of intravenous ribavirin (30 mg/kg initial dose, then 15 mg/kg every 6 h) within 4 h after exposure. Serologic follow-up up to 25 weeks showed no evidence of infection with Hendra virus.

Degree of exposure to infected horses and use of personal protective equipment (PPE) were assessed in 28 veterinary clinic staff. Twenty (including the 2 patients) reported contact with potentially infected equine body fluids; the Hendra virus attack rate for exposed staff was 10%. Fourteen identified high-risk exposures (potential exposure through unprotected mucous membrane, broken skin, or respiratory route), among whom self-reported use of PPE (respiratory droplet and mucosal protection) was low (7%). The other 6 staff identified low-risk exposures (intact skin). High-risk exposures identified among the 2 infected patients included performing daily nasal cavity lavage during the last 3 days of the incubation period on 1 infected horse (9 and 11 days before illness onset in patients 1 and 2, respectively) and participating in a necropsy of another infected horse (patient 1) 16 days before illness onset.

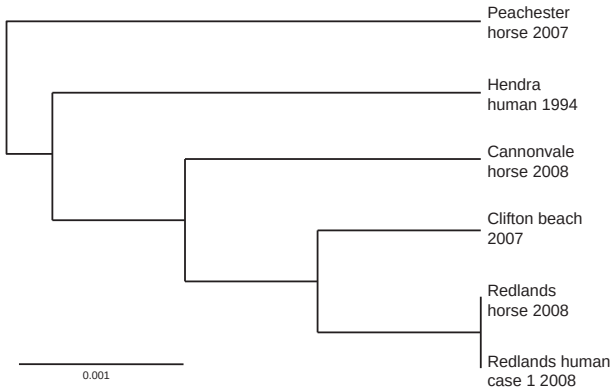

Figure 2. Phylogram showing relationships between Hendra virus isolates, Australia, 2008, based on medium gene sequence. Scale bar indicates nucleotide substitutions per site.

Discussion

Unlike previously recognized equine Hendra virus infections (*1*), this outbreak involved predominantly neurologic, rather than respiratory, equine symptoms. This finding may have contributed to delayed recognition of the etiologic cause among the sick horses and thus unprotected exposure of human contacts.

Of the 4 persons previously infected with Hendra virus, 1 died from multiorgan failure, another died from encephalitis 13 months after aseptic meningitis, and 2 others survived after ILIs. The 2 patients described in this report also had initial ILIs. However, soon after apparent clinical improvement, including defervescence, encephalitis developed in both patients. The MRI changes showed widespread cortical, subcortical, and deep white matter involvement, similar to those described in a previous Hendra virus encephalitis case (*3*) and Nipah virus encephalitis cases (*10*,*11*). These changes are consistent with the neuropathogenesis of *Henipavirus*-related encephalitis (widespread viral endothelial infection with resultant vasculitis, syncytial giant cell formation, and ischemic necrosis) (*12*). Although encephalitis developed in both patients 1–4 days after initial clinical improvement of the initial ILI and concurrent with the appearance of Hendra virus–specific antibodies, the ability of Henipaviruses to inhibit interferon signaling (*13*,*14*) is a major virulence factor that enables evasion of host responses. The other major phenomenon, described for 1 case of Hendra virus infection and in ≥5% and ≥10% of cases of Nipah virus encephalitic and nonencephalitic infections, respectively, is relapsed or late-onset encephalitic manifestations (*3*,*15*). However, the pathogenesis of this latency and understanding these clinical manifestations remain incomplete.

Given the previous demonstration of an in vitro effect of ribavirin against Hendra virus (*16*) and a reported survival benefit associated with its use (predominantly by the enteral route of administration) compared with historical controls in Nipah virus encephalitis (*17*), our patients

received treatment with this drug. For these patients, the high-dose intravenous regimen was used, which was similar to that used for patients with Lassa fever (*18*). Although anemia developed in patient 1, the drug was well tolerated by patient 2. Furthermore, given a previously reported case of late-onset Hendra virus encephalitis (*3*), patient 2 was given prolonged oral therapy (to month 8), which was well tolerated. The in vivo efficacy of ribavirin against Hendra virus remains unknown, although this drug delayed but did not prevent deaths caused by Nipah virus in a hamster model (*19*).

Although basal serum and CSF ribavirin concentrations ≥10 mg/L (during administration of ribavirin, 15 mg/kg every 6 h intravenously) and basal serum ribavirin concentrations of 6 mg/L (during administration of 8 mg/kg every 8 h orally) were documented, these concentrations may be inadequate given the results of in vitro susceptibility testing. The 90% inhibitory concentration of ribavirin against one of the human virus isolates was 64 mg/L, which was similar to that reported for an original human isolate from 1994 (*20*).

Treatment with ribavirin was initiated early (before onset of encephalitis) for patient 2 but later (upon onset of encephalitis) in patient 1. However, the influence of this timing on outcome remains unclear, particularly given the uncertainty about activity of ribavirin against Hendra virus and other possible confounding factors. For example, patient 1 had different and possibly greater quantitative initial exposure to Hendra virus, earlier onset of encephalitis, and corticosteroid therapy that may have suppressed virus clearance. We also administered intravenous ribavirin prophylactically for 5 days in another person after a percutaneous blood exposure to a horse documented as positive for Hendra virus by RT-PCR. This regimen was well tolerated, and follow-up assessments have not demonstrated seroconversion.

We emphasize that the efficacy of ribavirin as therapy or prophylaxis remains, at best, uncertain. However, given the absence of other available therapies, and the tolerability of ribavirin, we advocate that it be given early in clinical illness and be considered where high-risk exposure to Hendra virus has occurred. However, because only 6 cases have been recognized in humans, the criteria for high-risk exposure remain undefined.

This absence of established therapies underscores the need for early recognition of equine Hendra virus infection, implementation of infection control precautions, and prevention of horse-to-human transmission. Although the attack rate for humans exposed to infected horses in this outbreak (10%) was similar to that in the other documented stable outbreak that caused human infection (at which time the causative pathogen had not been identified) (*2*), upon recognition of the outbreak and implementation of protective measures, no further horse-to-human transmission occurred.

Because this outbreak involved hitherto unrecognized equine manifestations with predominant neurologic disease in the relative absence of respiratory signs, equine case definitions have been revised to include either respiratory or neurologic manifestations or both (*21*). The degree of nucleotide sequence variation observed between Hendra virus isolates from this and previous outbreaks suggests greater genomic variation than previously assumed. Whether the prominent encephalitic manifestations observed in this outbreak reflect this genetic variability require further assessment.

Detailed exposure histories from our 2 patients suggest that the likely incubation period was 9–16 days, which was longer than previously estimated. Furthermore, both patients performed nasal cavity lavage of a horse during the 3 days before its onset of symptoms of infection with Hendra virus. This finding suggests that horses may be infectious late in their incubation period, as observed with other Paramyxoviridae (e.g., measles).

The modes of horse-to-human transmission of Hendra virus are not known completely, but likely result from direct contact with respiratory secretions and other equine tissue and fluids or from droplet or aerosol exposure. Furthermore, difficulties associated with clinical recognition of equine Hendra virus infection present challenging infection control implications for equine veterinary practice. Therefore, routine adoption of infection control precautions encompassing hand washing, use of gloves and other forms of PPE, and a high index of clinical suspicion for equine Hendra virus infection are required. Although experience with human Hendra virus infection is relatively limited, no evidence exists of human-to-human transmission. However, persistence of Hendra virus RNA in NPA samples throughout the first 2 weeks of illness indicates that healthcare workers potentially exposed to respiratory secretions should adopt PPE use to prevent exposure to droplets.

In conclusion, we describe severe Hendra virus infection in 2 veterinary workers, 1 who died, after exposure to infected horses. Given the absence of other established therapies, ribavirin was administered. However, further understanding of the pathogenesis of neurologic disease is required to guide potential therapies. These evolving equine clinical manifestations and the inevitable and increasing interactions between the natural host of the virus (fruit bat species) and horses will continue to challenge veterinary, public health, and medical communities.

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Cost-effectiveness of Pharmaceutical-based Pandemic Influenza Mitigation Strategies[1]

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We used a hybrid transmission and economic model to evaluate the relative merits of stockpiling antiviral drugs and vaccine for pandemic influenza mitigation. In the absence of any intervention, our base-case assumptions generated a population clinical attack rate of 31.1%. For at least some parameter values, population prepandemic vaccination strategies were effective at containing an outbreak of pandemic influenza until the arrival of a matched vaccine. Because of the uncertain nature of many parameters, we used a probabilistic approach to determine the most cost-effective strategies. At a willingness to pay of >A$24,000 per life-year saved, more than half the simulations showed that a prepandemic vaccination program combined with antiviral treatment was cost-effective in Australia.

Influenza pandemics of varying severity occurred 3 times in the last century (1918, 1957, and 1968); the first influenza pandemic of the 21st century occurred in 2009. Before this latest pandemic, awareness had been heightened by the emergence of the highly pathogenic (H5N1) strain (*1*). In response, many countries have developed detailed plans aimed at the mitigation of a future pandemic. A key aspect of many pandemic plans is the stockpiling of antiviral drugs (neuraminidase inhibitors) for treatment or prophylaxis (*2*,*3*).

The stockpiling of prepandemic vaccine is also an area of active consideration (*4*). Although a matched vaccine (developed specifically for the emergent strain) is likely to offer the best protection, the delay in producing such a vaccine is a major obstacle. The stockpiling of prepandemic vaccine based on currently available strains avoids this delay but such vaccine is likely to provide lower efficacy than a matched vaccine. There is also a substantial risk that the pandemic strain will be of a different subtype than that chosen for the stockpiled vaccine. The emergence of pandemic (H1N1) 2009 illustrates this point.

Mathematical models of disease transmission have been used to assess the feasibility of pandemic mitigation strategies (*5*–*10*). However, of the limited numbers of published economic evaluations on pandemic stockpiling (*11*–*14*), to our knowledge only 1 recent study has attempted to directly model herd protection (*14*). We explored the cost-effectiveness of stockpiling prepandemic vaccine and antiviral drugs for pandemic influenza mitigation.

Methods

Overview

An age-stratified transmission model (susceptible, exposed, infected, removed) was used to calculate clinical attack rates (CAR) and antiviral drug consumption, which became inputs in a decision analytic economic model as represented in Figure 1 (MATLAB version 2008a [www.mathworks.com]). The primary outcome from the economic model was the incremental cost per life-year saved (LYS). Economic results are reported per person in the population to facilitate understanding for an international audience. We addressed the uncertainty in many of the model parameters by performing extensive sensitivity analyses, including probabilistic sensitivity analysis using 5,000 Latin hypercube samples drawn from parameter distributions. A detailed description of the transmission model and a full list of model parameters and distributions can be found

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[1]This material was compiled before the declaration of pandemic (H1N1) 2009 and concerns stockpiling for a future influenza pandemic.

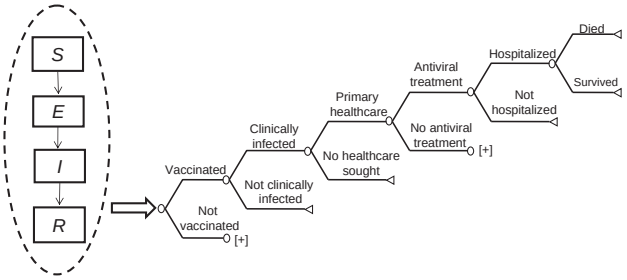

Figure 1. Schematic of hybrid transmission and decision analytic economic model. [+] indicates a cloned subtree with the same structure as the branch above. In sensitivity analysis, the probabilities of healthcare utilization and death were independent of each other but dependent on the probability of clinical infection. We assumed those with serious complications would seek primary healthcare. SEIR, susceptible, exposed, infected, removed.

in the appendices (online Technical Appendix 1, www.cdc.gov/EID/content/16/2/224-Techapp1.pdf, and online Technical Appendix 2, www.cdc.gov/EID/content/16/2/224-Techapp2.pdf).

Strategies

We considered prepandemic influenza vaccination in isolation and in combination with antiviral treatment. Four strategies for pandemic mitigation were examined (Table 1). In all strategies, a small stockpile of antiviral drugs was used for prophylaxis of case contacts and treatment of clinical cases in an initial containment effort and, after a delay of 6 months, a matched vaccine was delivered. In isolation, this intervention was labeled strategy 1.

Demographics

We divided the Australian population into 3 age groups: 0–19 years (26% [5,513,878], 20–64 years (61% [12,744,215], and ≥65 years (13% [2,759,129]) (*15*). Rates of mixing were age dependent and based on a recent large study of contact patterns in the European Union (*16*).

Vaccine Parameters

Immunogenicity data provide evidence of prepandemic vaccine efficacy (VE) in humans (*17*). However, this efficacy will also depend on how closely the prepandemic vaccine strain matches the pandemic strain that emerges. We assumed that 2 doses of prepandemic vaccine would reduce susceptibility (relative hazard of infection) by 40% in persons <65 years of age. For the ≥65 years of age group, VE may be reduced (*18*). Thus, in our base-case model, we halved the VE for this age group. Modeled efficacy was also dependent on the number of doses, time since the last dose, and vaccine type (prepandemic or matched).

The first dose of prepandemic vaccine was assumed to be given coincident with the first local case-patient, followed 21 days later by the second dose. In all strategies, the first dose of matched vaccine was provided 180 days after the first local case was identified in Australia; the second dose was administered 21 days later in strategies 1 and 2 only. Although 2 doses of a matched vaccine would be ordered under all strategies, in base-case only 1 dose of matched vaccine was administered in strategies involving 2 doses of a prepandemic vaccine (strategies 3 and 4). Population vaccine coverage (both prepandemic and matched) was assumed to be 80% (*19*).

Antiviral Drug Parameters

In the base-case model, we estimated the efficacy of antiviral treatment for preventing hospitalization as 59% (*20*). We assumed the same antiviral drug efficacy for preventing death as for hospitalization. The effect of antiviral treatment on influenza transmission in the community is unclear (*21*), and we assumed no reduction in infectivity of treated cases. When antiviral treatment was given, it was provided to 80% of persons with clinical disease (those who sought primary healthcare). Antiviral drug strategies contained stockpiles to cover 40% of the population (≈8 million courses). However, all strategies assumed a limited antiviral drug stockpile (≈0.2 million courses) to be used in initial containment efforts for treatment of clinical cases and prophylaxis of case contacts. We assumed that the antiviral prophylaxis treatment of contacts, during the initial containment effort, reduced susceptibility and infectiousness by 70% and 60%, respectively (*22*). A static percentage (10%) of viruses were assumed resistant to antiviral drugs.

Disease Estimates

The CAR during a pandemic was determined by using the transmission model and was primarily a function of R_0 (1.7) (*23–25*) and the percentage of asymptomatic infections (50%) (*26*). The basic reproductive number (R_0) represents the number of secondary cases that a representative person with influenza would infect in a fully susceptible population. Asymptomatic persons were assumed to be two thirds as infectious as symptomatic persons (*9*,*10*). We assumed that 50% of those with a clinical influenza infection would seek medical care (*27*); most primary care would occur in general practice (80%) and the remainder in hospital emergency departments.

Table 1. Descriptions of 4 pharmaceutical-based pandemic influenza mitigation strategies*

Strategy no.	Description
1	Minimum pharmaceutical intervention
2	Antiviral treatment of those clinically infected
3	Population prepandemic vaccination
4	Strategies 2 + 3

*All strategies included an initial antiviral containment effort and distribution of a matched vaccine to the population 180 days after the first locally acquired case.

The rates of hospitalization and death were defined relative to the CAR by using a case-hospitalization rate and case-fatality rate. We used age-specific case-hospitalization rates (0–19 years = 1.875%, 20–64 years = 2.5%, ≥65 years = 5%) and case-fatality rates (0–19 years = 0.75%, 20–64 years = 1%, ≥65 years = 2%). There are no reliable estimates of when a future pandemic might occur. We used a base-case delay to a pandemic of 5 years.

Cost Estimates

As recommended by Australian pharmaceutical funding guidelines, we focused on direct healthcare costs (*28*) and performed our base-case analysis from a healthcare system perspective. In scenario analysis we considered a broader societal perspective, which included lost production costs. Costs and effects were discounted at 5% annually (*28*). All costs are reported in 2005 Australian dollars.

Intervention Costs

In the base-case model, we assumed a stockpile purchase price for pharmaceuticals of $12 per vaccine dose and $32 per antiviral course; a range of values was considered in sensitivity analysis. The limited shelf-life of the pharmaceuticals requires the renewal of stockpiles for prepandemic vaccine every 3 years and antiviral drugs every 5 years. The number of times stockpiles were replaced was based on the expected time to a pandemic. We assumed partial replacement of the stockpile annually on a continuous basis. An annual storage cost for vaccines ($1, refrigeration) and antiviral drugs ($0.5, no refrigeration) was included.

We assumed that vaccination (and initial antiviral drug distribution) would be administered in mass clinics at a cost of $11.60 per course/dose (*29*). An administration cost for antiviral treatment was not included because this treatment would be given as part of a primary care visit for influenza illness. However, for strategies that included antiviral treatment, the percentage of clinical cases seeking medical care was increased to 80%.

Healthcare Unit Costs

Hospitalization costs were based on analysis previously conducted by our group, which reviewed records of patients hospitalized for influenza and pneumonia in Australia (*30*). We estimated age-specific hospitalization costs by multiplying the average cost per day by the average length of stay for that age group (*31*). Expenses for emergency department visits for influenza not requiring hospitalization were estimated by the Australian Ambulatory Classes emergency department presentation cost for "Other respiratory diseases with procedure" (*32*). The cost of a general practitioner visit for influenza ($33.32) was based on a general practitioner survey of consultation for influenza-like illness (*30*).

Production Costs (Societal Perspective Only)

The costs of lost production were valued by using the human capital approach. Lost production was only valued for those employed in paid work (*33*). The cost attached to lost work days was based on average weekly earnings (*34*). Clinical influenza patients were assumed to have 2.6 days absent from work (*35*). We assumed that those <15 years of age would require 1 adult caregiver when sick. We used length of stay to estimate lost production for hospitalized patients.

Results

Clinical Outcomes

The base-case analysis used an R_0 value of 1.7, which led to a CAR of 31.1% in the overall population in the absence of any intervention. The assumption of greater mixing in children meant that this group experienced the highest CARs, with 38.1% in persons 0–19 years of age, 30.4% in persons 20–64 years of age, and 20.4% in persons ≥65 years of age. In the absence of any intervention, the base-case model produced an overall population hospitalization rate of 782.3/100,000 persons and a mortality rate of 312.9/100,000. Strategies incorporating a population prepandemic vaccination program resulted in a low CAR and, consequently, a low number of hospitalizations (strategy 3 = 136.8/100,000; strategy 4 = 79.4/100,000) and deaths (strategy 3 = 54.7/100,000; strategy 4 = 31.8/100,000). The antiviral drug treatment strategy did not affect the CAR but significantly reduced the number of hospitalizations (strategy 2 = 450.0/100,000) and deaths (strategy 2 = 180.0/100,000).

Several parameters were influential in determining the CARs (Figure 2). In prepandemic vaccination strategies, R_0 (Figure 2, panel A), VE (Figure 2, panel A), and vaccine coverage (Figure 2, panel B) played major roles in determining whether a large outbreak was prevented or simply mitigated. The CAR rose as R_0 increased and declined as VE improved and coverage increased, with sharper transitions occurring as the number of secondary cases that a single case infects approached 1. The CAR for prepandemic vaccination strategies also increased markedly when vaccination was delayed until after a local outbreak had commenced (Figure 2, panel C). The number of deaths prevented by antiviral treatment rises as R_0 increases (Figure 2, panel D). This increase occurs because the incidence of preventable disease is larger for higher values of R_0. The effect on prepandemic vaccination strategies is similar, provided the strategy is largely successful in containing the pandemic. However, for R_0 values >1.7, when this is no longer the case, prepandemic vaccinations strategies prevented fewer deaths (Figure 2, panel D).

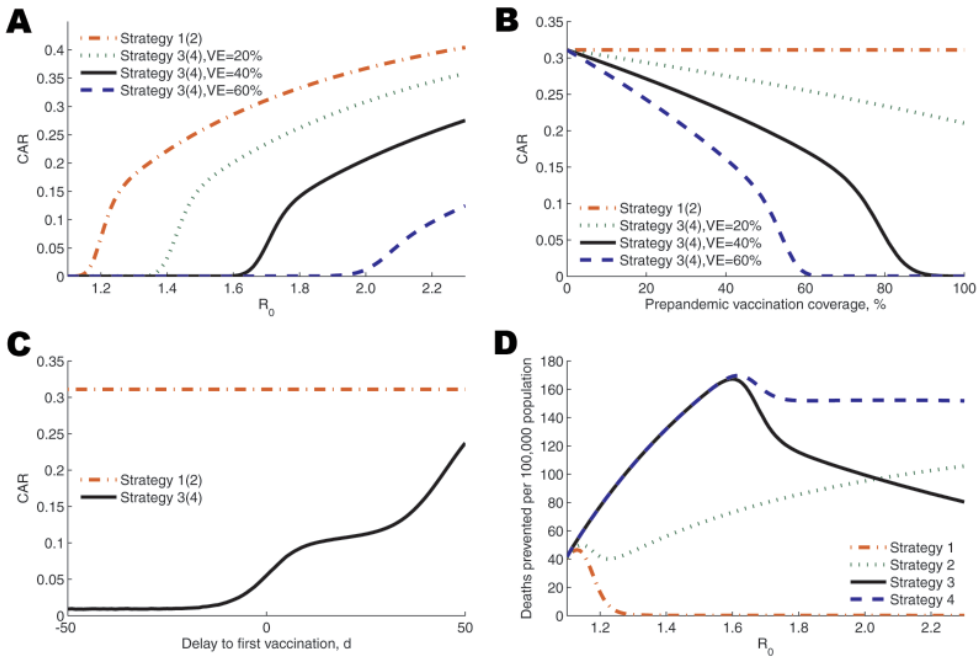

Figure 2. Sensitivity analyses of clinical outcomes as key parameters are varied. In A–C, the clinical attack rate (CAR) is displayed as a function of R_0 and vaccine efficacy (VE) (A), vaccine coverage and VE (B), and the delay to vaccination (C). In D, deaths prevented per 100,000 population compared with no intervention is displayed as a function of R_0.

Economic Outcomes

The total discounted healthcare costs for a pandemic in the absence of any intervention was $31.1/person in the population. The gross discounted cost over 5 years (including purchase, replacement, storage, and administration) of a prepandemic vaccination program was $68.4/person in the population and the cost of an antiviral treatment program (purchase, replacement, and storage only) over the same period was $24.8/person.

The base-case results for the healthcare system perspective are shown in Table 2. In the base-case model (R_0 = 1.7, VE = 40%), strategies 2–4 each offered increased effectiveness at an increased cost when compared with the next best strategy. Under these conditions (theoretically), decision makers should first decide if strategy 2 offers value for money (incremental cost-effectiveness ratio [ICER] = $909/LYS) and then consider the value offered by each additional increase in spending, moving from strategy 2 to 3 (ICER = $1,084/LYS) and then from strategy 3 to 4 (ICER = $7,458/LYS).

From a societal perspective the least costly strategy was prepandemic vaccination (strategy 3), which was cost saving when compared with the minimum pharmaceutical intervention. Strategy 3 also dominated antiviral drug treatment alone (strategy 2), being more effective and less costly. The addition of antiviral drug treatment to prepandemic vaccination cost $7,404/LYS.

Sensitivity Analyses (Healthcare System Perspective)

Key parameters affecting the cost-effectiveness of strategies included the R_0 value and factors impacting vaccine or antiviral effectiveness. Because strategies differed in their sensitivity to these parameters, the cost-effectiveness of strategies relative to each other varied. Dominance occurs when a strategy is considered superior to the alternative by being either more effective and less costly (simple dominance) or more effective and more costly but with a lower ICER (extended dominance) (*36*). At higher values of VE (>41%) or when the percentage of antiviral given within 48 h was <75% (base-case = 80%), prepandemic vaccination dominated antiviral drug treatment alone. When the VE was >50% or the R_0 <1.6, prepandemic vaccination alone was largely sufficient to contain the pandemic, and the addition of antiviral treatment offered only a minimal incremental effect at a high incremental cost (ICER >$1million

Table 2. Base-case economic results per person in the population (healthcare system perspective) of 4 pharmaceutical-based pandemic influenza mitigation strategies*

Strategy no.	Net cost	Incremental cost	LYS	Incremental LYS	Incremental cost per LYS†
1	65.88	–	–	–	–
2	82.24	16.36	0.01803	0.01803	908
3	100.65	18.40	0.03501	0.01698	1,084
4	124.00	23.36	0.03814	0.00313	7,458

*LYS, life-year saved. Costs and life-years discounted at 5% annually; all costs calculated in 2005 Australian dollars.
†Rounded to the nearest whole dollar.

per LYS). At lower values of VE (37%) or at higher values of R_0 (1.8), prepandemic vaccination alone was dominated by prepandemic vaccination combined with antiviral drug treatment, which offered reasonable value for money (ICER <$3,500/LYS) when compared with antiviral drug treatment alone. When we considered a VE of 20%, the addition of prepandemic vaccination to antiviral treatment alone cost ≈$9,000/LYS.

To be cost saving, prepandemic vaccine (strategy 3) and antiviral drug treatment (strategy 2) would have to be priced at <$3.1/dose and <$10.0/course when compared with the minimum pharmaceutical intervention. Variation in most other parameters did not affect the cost-effectiveness of strategies relative to each other. When the CAR was reduced (20% in the absence of any intervention) as a result of the percentage of asymptomatic infections, the ICER of all strategies increased. However, all strategies still had an ICER <$10,000/LYS. When we assumed the pandemic was relatively mild (case-fatality and case-hospitalization rates 5× less than base-case) and occurred 30 years later, all strategies had ICERs >$50,000/LYS. Varying the age distribution of severe clinical cases (case-fatality and case-hospitalization cases) had only a minor impact on the cost-effectiveness. When we varied the discounting rate (to be either 0% or 3% for costs and effects), ICER for all strategies decreased with no change to strategy order. Variation in other parameters was explored in probabilistic sensitivity analysis.

Probabilistic Sensitivity Analyses

Cost-effectiveness acceptability curves (Figure 3) enable decision makers to estimate the probability that a strategy is optimal as a function of their willingness to pay for additional units of effect. At decision makers' willingness to pay >$24,000/LYS, more than half of simulations found that a prepandemic vaccination program combined with antiviral treatment was cost-effective in Australia (Figure 3, panels A and C). However, when we assumed that half of the time the emergent pandemic strain would have a different subtype than that chosen for the stockpiled vaccine (Figure 3, panels B and D), most simulations (willingness to pay >$12,000/LYS) found that antiviral drug treatment alone was the optimal strategy.

Discussion

Under the assumption of a severe pandemic occurring in the near future, the pharmaceutical-based mitigation strategies examined were generally estimated to be cost-effective. For at least some of the plausible range of transmission parameters, strategies involving population prepandemic vaccination were effective in containing an outbreak until the arrival of a matched vaccine. A combination of antiviral drug treatment and prepandemic vaccination offered the best protection for the population. From a societal perspective, prepandemic vaccination was estimated to be cost saving when compared with the minimum pharmaceutical intervention.

The cost-effectiveness of pandemic influenza mitigation strategies was quite resilient to major changes in influential parameters such as the value of R_0 and the effectiveness of vaccination and antiviral drugs. This resilience stems from 2 important assumptions: 1) we assumed that the pandemic would be severe (our base-case has similar characteristics to the 1918 pandemic); and 2) we assumed a pandemic would

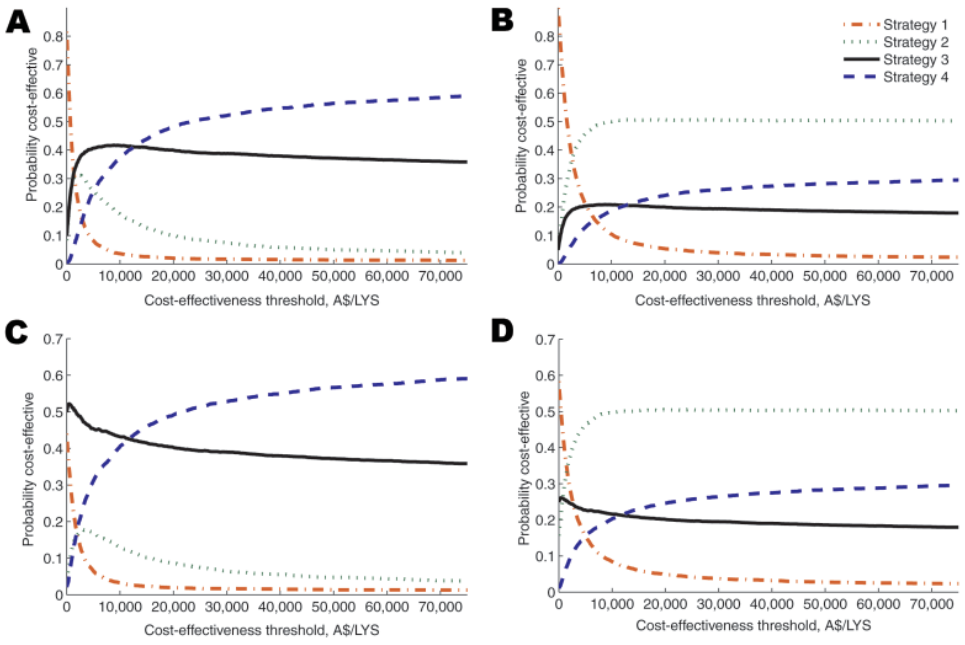

Figure 3. Cost-effectiveness acceptability curves. Panels A and B show the healthcare system perspective; C and D show the societal perspective. In B and D, we assumed that half of the time (Q = 50%) the emergent pandemic strain would be would be of a subtype to which the stockpiled vaccine offered no protection. We did not explore the use of such a vaccine in subsequent pandemics. Costs and life-years discounted at 5% annually. A$, Australian dollars; LYS, life-year saved.

occur soon (5-year delay in base-case). The first assumption implies that the consequences of a pandemic would be large in terms of the number of deaths and the healthcare resources required, whereas the second assumption implies that the costs associated with maintaining a stockpile were limited and that the future benefits would not be dramatically reduced by discounting. Under these assumptions, even moderately effective interventions from a clinical perspective (e.g., a vaccine with 20% efficacy) may be cost-effective. When we assumed instead that the pandemic was relatively mild (case-fatality and case-hospitalization rates 5× less than base-case) and occurred 30 years later, pandemic mitigation strategies were borderline cost-effective at best. This mild scenario still assumes a disease incidence several times that of seasonal influenza.

We found that vaccination and antiviral strategies differed in their sensitivity to certain key parameters (Figure 2). Because the value for value for money offered by the intervention strategies was relatively similar, even minor changes in some parameters led strategies to become dominated by a more effective alternative. For example, when VE increased above 41% (base-case 40%), antiviral drug treatment alone was dominated (extended) by prepandemic vaccination (strategy 3). This sensitivity highlights the inadequacy of a base-case analysis and the need for probabilistic sensitivity analysis (Figure 3).

This analysis was restricted by a lack of accurate information on prepandemic VE. However, because any emergent pandemic strain is unknown, some level of uncertainty around VE is unavoidable. We assumed that a prepandemic vaccine would offer moderate protection (below that of a matched seasonal vaccine), and using probabilistic sensitivity analysis (Figure 3, panels B and D), we explored the risk that the emergent pandemic strain would be of a different subtype than that chosen for the stockpiled vaccine. We did not specifically explore the use of such a vaccine in subsequent future pandemics or the separate stockpiling of adjuvant and antigen. The effectiveness of antiviral drugs can also not be known with certainty in advance. We assumed a static 10% resistance to antiviral drugs and varied this widely in sensitivity analysis. A more realistic model would take into consideration possible development of resistance over time (*37*), but a detailed analysis of antiviral resistance was beyond the scope of our analysis.

Our model approach was deterministic so that although stochastic variation in parameters was considered, identical parameter choices led to identical model outputs. Because our analysis was limited to assessing the effect on overall attack rates and the costs and benefits associated with this, rather than outputs such as daily case counts, the influence of stochasticity at the simulation level should be relatively minor. Furthermore, the importation of cases from outside the country is likely to rapidly increase counts to a level at which deterministic behavior dominates. A major advantage of a simple deterministic approach is that sensitivity analyses are not constrained by computational resources, enabling detailed uncertainty analysis.

We have largely ignored issues of capacity constraint. For instance, hospital bed day capacity is likely to be severely strained during the peak of an influenza pandemic (*38*). A severe influenza pandemic is also likely to have a dramatic effect on the broader economy (*39*), which may not be captured well even under our societal perspective. Studies estimating the macroeconomic impact of a pandemic are beginning to emerge (*40*). The failure to capture the broader macroeconomic impact makes our healthcare system perspective conservative. However, the extent to which the benefits are captured (or not captured) is likely to be different for each strategy.

Population prepandemic vaccine and antiviral drug treatment strategies offer substantial scope to be cost-effective strategies for pandemic influenza mitigation. Unlike antiviral treatment strategies, population prepandemic vaccination offers the possibility of containment until the arrival of a matched vaccine. The stockpiling of prepandemic vaccines should be carefully considered and take into account the current level of uncertainty and budgetary limitations.

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Domestic Animals and Epidemiology of Visceral Leishmaniasis, Nepal

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On the Indian subcontinent, visceral leishmaniasis (VL) is considered an anthroponosis. To determine possible reasons for its persistence during interepidemic periods, we mapped *Leishmania* infections among healthy persons and animals in an area of active VL transmission in Nepal. During 4 months (September 2007–February 2008), blood was collected from persons, goats, cows, and buffaloes in 1 village. *Leishmania* infections were determined by using PCR. We found infections among persons (6.1%), cows (5%), buffaloes (4%), and goats (16%). Data were georeferenced and entered into a geographic information system. The bivariate K-function results indicated spatial clustering of *Leishmania* spp.–positive persons and domestic animals. Classification tree analysis determined that among several possible risk factors for *Leishmania* infection among persons, proximity of *Leishmania* spp.–positive goats ranked first. Although our data do not necessarily mean that goats constitute a reservoir host of *L. donovani*, these observations indicate the need for further investigation of goats' possible role in VL transmission.

Visceral leishmaniasis (VL), also known as kala-azar, is a fatal vector-borne parasitic disease. Worldwide incidence is 500,000 cases per year; ≈90% of cases occur in India, Nepal, Bangladesh, Sudan, and Brazil (*1*). On the Indian subcontinent, the number of officially reported cases, although only a fraction of the true incidence (*2*), has increased during the past 5–6 years, and the disease is spreading to new areas (*3*). A kala-azar elimination program was recently launched by the governments of Bangladesh, India, and Nepal, with the support of the World Health Organization; the goal is to reduce the annual incidence in VL-in endemic regions to <1 case per 10,000 persons by 2015 (*4*). The program essentially relies on early diagnosis and treatment of persons and on vector control (*5*). This strategy is based on the assumption that *Leishmania donovani*, the etiologic agent of VL, is transmitted from person to person (anthroponotic VL).

The possible role of domestic animals in anthroponotic VL has been studied in Bangladesh (*6*), but no clear conclusions have been drawn with regard to animals as risk factors or reservoir hosts. In contrast, the proximity to a VL-infected person is a major risk factor for VL (*6*). Thus, persons are still considered the only reservoir host for *L. donovani* on the Indian subcontinent. However, the reasons for persistence during interepidemic periods are debated, and dermal leishmaniasis after kala-azar has been incriminated (*7*). Correct identification of the *Leishmania* reservoir host is crucial for the design of control programs. Molecular tools offer new opportunities to better document and reassess transmission patterns. To explore the potential role of domestic animals in transmission, we performed an extensive study in an area of active VL transmission in Nepal, mapping *Leishmania* infections among healthy persons and domestic animals.

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[1]Deceased. This article is dedicated to his memory.

Materials and Methods

Study Site

The study was conducted as part of the KALANET project, a community trial of insecticide-treated bed nets (www.kalanetproject.org) in the Terai region of eastern Nepal. In Nepal, each village is divided into several wards. For the KALANET project, 10 wards with active VL transmission were selected. Dharan-17 was 1 such ward; it is a periurban ward in the Dharan municipality, located in the foothills of the Mahabharata hills and along the bank of Sardu River. Dharan-17 covers ≈0.3 km^2 (Figure 1) and has 515 inhabitants living in 105 households (Figure 2). A demographic survey conducted in July 2006 showed that 77% of households had at least 1 domestic animal (i.e., cow, goat, dog). Most cows, buffaloes, and goats were kept <10 m from the households at night, although a few goats were kept inside the house. VL was only recently reported in this periurban area; Dharan-17 has an average VL incidence rate of 1.61% per year (for 2004–2006). Furthermore, during a previous study conducted in 2006, we documented a higher rate of VL positivity by PCR among the healthy persons in Dharan-17 compared with those in the 9 other wards in the KALANET trial, possibly suggesting a high transmission rate (*8*). For a control area, we selected Dhankura-3 in Patlekhola. This ward is ≈60 km from Dharan-17, in a hilly area where no VL cases have yet been reported.

Ethical Aspects

Ethical clearance for the KALANET project was obtained from the Ethical Committee of the B.P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal, and the corresponding bodies at the Institute of Tropical Medicine, Antwerp, Belgium, and the London School of Hygiene and Tropical Medicine, London, UK. A community meeting informed local leaders and village residents about the study purpose; informed consent was obtained from all animal owners before their animals were included in the study. International animal experimentation guidelines were followed. Persons provided written consent before enrollment and providing blood samples, per the human experimentation guidelines approved by BPKIHS and the corresponding body at the Institute of Tropical Medicine, Antwerp. For religious reasons, 10.67% of persons did not provide written consent to donate their blood samples.

Sample Collection

All animal surveys were conducted by experienced veterinarians. In Dharan-17, a house-to-house survey was first conducted in September 2007, among 105 households, to collect information on the number and types of animals present in the ward; only information about bovines (cows and buffaloes) and goats was collected in this first survey. Later, 2 sampling surveys were conducted in this ward. In October 2007, survey I sampled 144 goats, 24 buffaloes, and 20 cows from the 37 households that had ≥1 bovine or goat. In February 2008, survey II focused on 6 households in which *Leishmania* spp.–positive

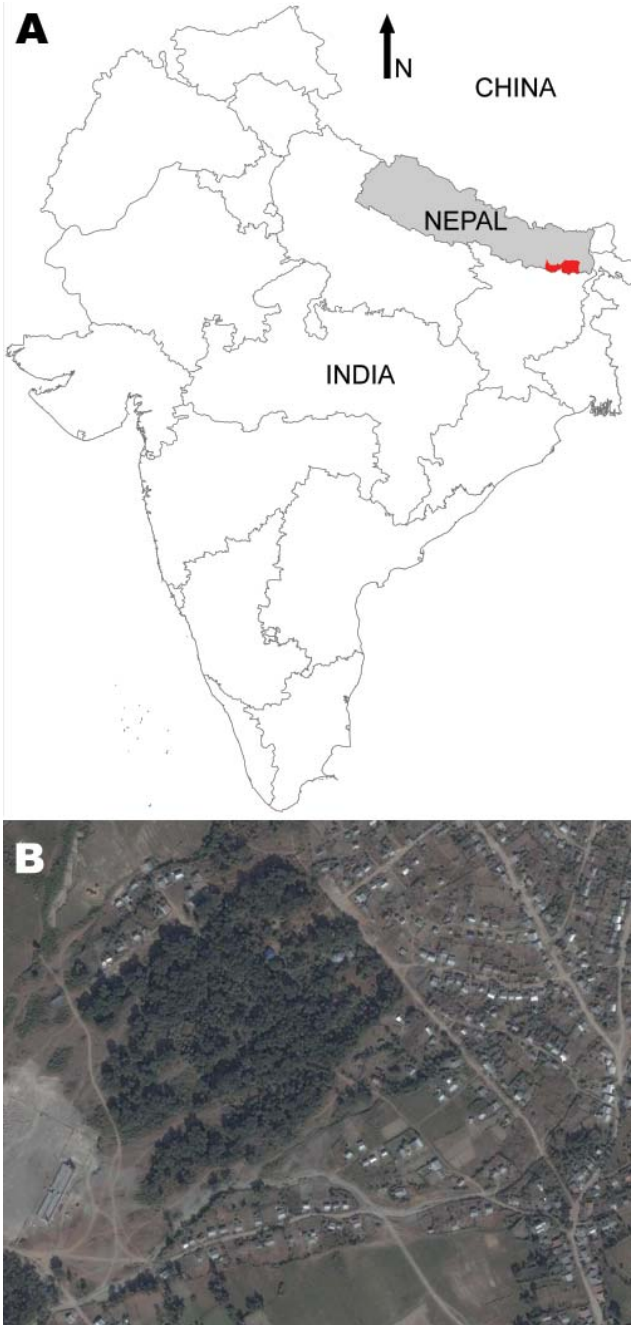

Figure 1. A) Visceral leishmaniasis–endemic area (red) of Nepal under study by KALANET project (www.kalanetproject.org); B) satellite picture of Dharan-17, Nepal. Copyrights 2009 Google Image; 2009 DigitalGlobe; 2009 Europa Technologies; and 2009 Mapabc.com.

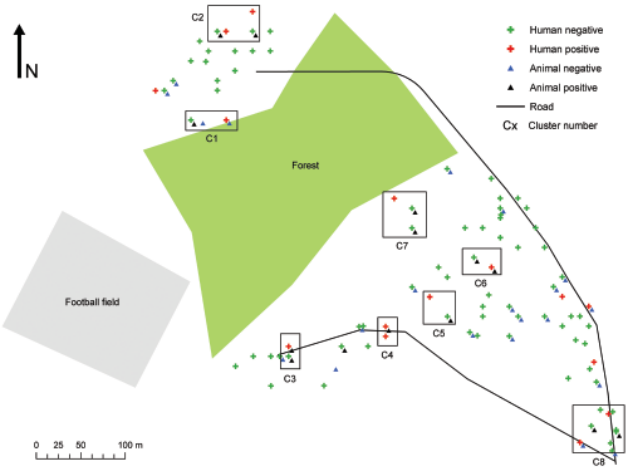

Figure 2. Distribution of sampled households and domestic animals, by visceral leishmaniasis status as determined by PCR, Dharan-17, Nepal, September 2007–February 2008.

animals had been identified during survey I. Although the owners claimed that the goats in surveys I and II were the same goats, we could not confirm this. In February 2008, samples were collected from 25 goats, 17 buffaloes, and 21 cows in the control area. In addition to animal samples, we also collected 278 blood samples from persons, all >5 years of age, who lived in Dharan-17 at the time of the survey and provided consent. The samples (1 mL) were collected by venipuncture from animals and persons into tubes containing molecular biology grade Na_2EDTA (240 μg/mL of blood; Sigma-Aldrich, Bornem, Belgium). All tubes were immediately stored in a chilled ice box and transferred on the same day to the laboratory at BPKIHS, where 180 μL of each sample was transferred to a tube containing 180 μL of AS1 buffer (catalog no. 1006243; QIAGEN, Venlo, the Netherlands), mixed well, and stored at room temperature.

DNA Extraction and PCR Amplification

All blood samples stored in AS1 buffer were used to extract the DNA within 1 month. The QIAamp DNA Mini Kit (catalog no. 56301; QIAGEN) was used to extract DNA at BPKIHS, following manufacturer's instructions. All DNA samples were sent at ambient temperature to the Institute of Tropical Medicine, Antwerp, where they were analyzed by PCR specific for small ribosomal genes of *Leishmania* spp. as described elsewhere (*9*). To confirm that the amplified DNA corresponded to *Leishmania* spp., amplicons from a set of positive samples were sequenced. The sequences were compared with those of *Leishmania* spp. and other trypanosomatids from GenBank.

Spatial Clustering of *Leishmania* spp.–positive Households

We used the results from survey I to assess the clustering of the *Leishmania* spp.–positive samples in Dharan-17. Each household, previously georeferenced by a geographic positioning system and mapped by using ArcGIS 9.2 (ESRI, Redlands, CA, USA), was identified as *Leishmania* spp.–positive or –negative for animal and human samples. Analyses considered all animals (goats, cows, and buffaloes) together. The bivariate K-function was used to determine whether households with *Leishmania* spp.–positive persons were spatially clustered around households with *Leishmania* spp.–positive domestic animals in Dharan-17. The following equation was used (*10*): K(d) = expected no. events B within distance d of arbitrary event A / overall density of events B. For easier interpretation of the results, the bivariate K-function was transformed in an L-function as follows (*11*):

$$L(d) = \sqrt{\frac{K(d)}{\pi}} - d$$

Positive L(d) results would suggest that human and animal *Leishmania* spp.–positive households are spatially associated. Further technical details on the bivariate K-function are available in the online Technical Appendix (www.cdc.gov/EID/content/16/2/231-Techapp.pdf) (*10–12*).

Classification Trees

Classification trees (CTs) and regression trees (Salford Systems, San Diego, CA, USA) can be used in classification and regression problems (*13*). We used CTs to analyze risk factors and identify interactions for *Leishmania* spp.–positive households. This nonlinear, assumption-free, and algorithm-based method splits data variance across nested nodes (with increased importance toward the tree base). Its algorithm automatically eliminates variables without explanatory power. Sensitivity (% true positives) and specificity (% true negatives) were computed. A 10-fold cross-validation (leaving 10% of the data at a time for the whole dataset to compute percentage of misclassifications) was used. Node impurity was measured by using the Gini index, and the minimal size of the base and terminal nodes were set to 10 and 1 cases, respectively. The method is explained in more detail elsewhere (online Technical Appendix) (*14–17*). The factors included in the CT analysis were 1) household (i.e., type of house, head of household occupation/education) and biologic (i.e., density of domestic animals around households, collected in a house-to-house survey in July 2006 and estimated by using a kernel density function [ArcGIS, ESRI]) (*6*) and 2) results of PCR analyses (Table 1).

Table 1. Risk factors included in classification tree analysis of visceral leishmaniasis in Nepal*

Biological factors (animals)
No./household
Samples from buffalo, cow, or goat
Leishmania spp.–positive samples from buffalo, cow, or goat
No. households with *Leishmania* spp.–positive animals 10, 25, 50, or 100 m from house
Minimum, mean, and maximum distance to household with *Leishmania* spp.– positive animal
Nos. poultry, birds, poultry and birds, pigs, dogs
Density (no./km^2) of poultry, birds, poultry and birds, buffaloes, cows, goats, pigs
Biological factors (humans)
No./household
Samples for PCR
Leishmania spp.–positive samples
No. households with *Leishmania* spp.–positive persons 10, 25, 50, or 100 m from house
Dichotomous variable for positive households for humans
Minimum, mean, and maximum distance to household with *Leishmania* spp.–positive person
Household factors
No. houses, identity of household, latitude, longitude, education of head of household, occupation of head of household, category house type, socioeconomic index
No. persons/ household, rooms, persons/room, bed nets, bed nets/person

*More details available upon request from J.-C.D.

Results

Animal Sample PCR Results

Survey I found 188 domestic animals (goats, buffaloes, and cows) in only 37 of the 105 households of Dharan-17. The overall rate of *Leishmania* positivity was 13.3% (25/188); goats accounted for 16% (23/144), cows for 5% (1/20), and buffaloes for 4% (1/24).

The *Leishmania* spp.–positive animals were encountered in the 15 households shown in Figure 2. During survey II, only goats were sampled from 6 of these households; 2 goats (from different households) of 24 (8.34%) were *Leishmania* spp. positive. All sequenced amplicons confirmed the presence of a *Leishmania*-specific sequence. All 63 samples from animals in the control area were negative.

Human Sample PCR Results and Spatial Clustering

In Dharan-17, of the 278 persons sampled, 17 (6.1%) were *Leishmania* spp. positive, 14 were healthy with no history of kala-azar, and none was a household contact of a VL case-patient. Of the 17 *Leishmania* spp.–positive persons, 2 had had VL and had been successfully treated. We could not determine the history for 1 person because he had moved out of the ward. The 17 *Leishmania* spp.–positive persons were from 16 households. The Kuldorff spatial scan statistic (*18*) was used to assess whether *Leishmania* spp.–positive persons were clustered in Dharan-17, but no significant clusters were detected. Analogous results were obtained for *Leishmania* spp.–positive animals (results not shown). However, when we superimposed households and *Leishmania* spp.–positive persons or animals (Figure 2), it visually appeared that 1) in 8 sites of the ward, *Leishmania* spp.–positive persons were localized in households near where *Leishmania* spp.–positive animals were kept (distance between the households <30 m; further called clusters), and that 2) in 1 site only *Leishmania* spp.–positive persons were found (Table 2). Some clusters constituted hot spots (had several cases of infection): clusters 2 (9 animals and 2 persons) and 8 (6 animals and 2 persons). The clusters represented in Figure 2 and detailed in Table 2 were determined visually; no statistical methods were applied. The bivariate K-function results show that households with *Leishmania* spp.–positive persons were clustered around households with *Leishmania* spp.–positive animals; $L(d)$ is positive from 0 to 100 m. However, the spatial association between them is only significant from 0 to 5 m (online Technical Appendix).

Classification Tree Analysis

The bivariate K-function analyzes only the relationship between *Leishmania* spp.–positive households for persons and domestic animals in Dharan. The results of this function have no meaning other than a spatial grouping of households with positive animals and persons. In a second stage, we used a CT model to analyze risk factors for *Leishmania* spp.–positive households. First we analyzed households in which *Leishmania* spp.–positive persons had been encountered. These results showed that the minimum distance to a household with a *Leishmania* spp.–positive animal (any species) was the variable with the highest discriminatory power. It appears first in the tree (Figure 3, panel A) and gets a relative importance score of 100% (data not shown). Discriminating distance was 22.8 m: households <22.8 m from a household with

Table 2. Distribution of *Leishmania* spp.–positive cases of visceral leishmaniasis among clusters, Nepal, September 2007–February 2008*

Cluster	No. animals	No. humans
C1	1	1
C2	9	2
C3	2	1
C4	1	4
C5	1	1
C6	3	1
C7	3	1
C8	6	2
Out of cluster	1	4

*Cluster group of households situated near (<30 m) each other and in which *Leishmania* spp.– positive animals or humans were encountered.

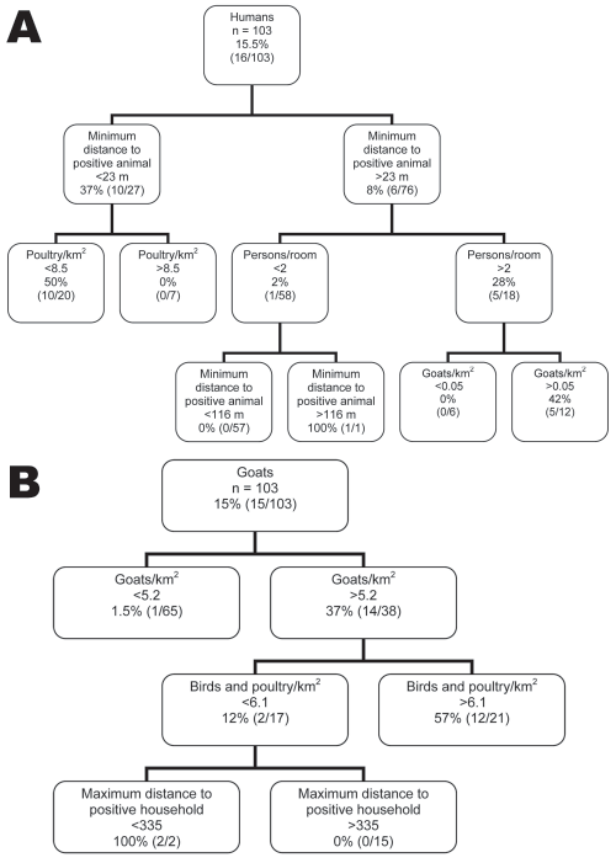

Figure 3. Classification tree results, showing interplay between risk factors of *Leishmania* positivity, determined by PCR, for A) humans and B) goats, in Dharan-17, Nepal, September 2007–February 2008.

Leishmania spp.–positive animals showed a 37% probability of hosting *Leishmania* spp.–positive persons versus 7.9% if they were >22.8 m from a household with *Leishmania* spp.–positive animals. The next variables appearing on the tree were the density in poultry (higher risk for *Leishmania* spp.–positive persons if density ≤8.55/km^2), the number of persons per room (higher risk if >2.88 persons/room), and density of goats (higher risk if >0.05 goats/ km^2). A second CT analysis was conducted for households in which *Leishmania* spp.–positive animals had been encountered. The generated tree differed from the previous one in that the first discriminating variable was the density of goats per km^2; risk for *Leishmania* spp. positivity associated with a density >5.2 (36.8% vs. 1.5%; Figure 3, panel B) was higher, and a relative importance score was 100% (data not shown). The next variables appearing on the tree were the density of birds and poultry per km^2 (higher risk for *Leishmania* spp.–positive goats if >6.14) and the maximum distance to a household with a *Leishmania* spp.–positive person (higher risk if <334.6 m). All these variables also appeared with highest predictor ranking scores (data not shown).

For the tree in Figure 3, panel A, the sensitivity and specificity of the tree were 100% and 80.5%, respectively. For the tree in Figure 3, panel B, sensitivity and specificity were 93.3% and 89.8%, respectively.

The minimum distance to a positive animal split in Figure 3, panel A, is difficult to interpret. It involves 1 positive result and is under a branch that was determined to be predominantly negative as a result of the first split when using the same variable at >23 m. This split should therefore be interpreted with caution. The same applies for the split of maximum distance to positive household ≤335 m (Figure 3, panel B), which is difficult to interpret if the study area is only 300 m^2 and may be the result of the irregular shape of the study area.

Discussion

We found *Leishmania* DNA in domestic animals (cows, buffaloes, and goats) from Dharan-17, mostly in goats (16%), although no goats or other animals in the control area were *Leishmania* spp. positive. As DNA persists in the body for only a short time (24 h) (*19*), PCR positivity is a good indicator of current (or recent) infection. Considering the time between the *Leishmania* spp. peak transmission season in Nepal (estimated in April–May) (*20*) and our first survey (October 2007), our results thus indicate that goats can be infected with *L. donovani* and that this infection persists for at least several months. Contact with *Leishmania* organisms, as shown by serologic findings, has already been reported for goats and other domestic animals in Sudan (*21*).

The decrease in *Leishmania* spp. positivity between the 2 surveys (from 16% in October 2007 to 8.34% in February 2008) could be explained by several reasons: 1) sampling bias, 2) effective decrease of parasitemia over a certain period because of immunologic control of infection, or 3) disappearance of positive animals from the ward. A follow-up study of *Leishmania* spp.–infected goats (up to at least 12 months) combined with an adequate tracking system are needed to determine whether parasites are still in the blood of the animals during the next transmission peak.

Comparison of the results from animals with those from healthy human volunteers from Dharan-17 provided additional interpretation of the animal results. *Leishmania* spp. positivity was found to be ≈3× lower among persons than among animals (6.1% vs. 16%, respectively). These data are consistent with data on the feeding behavior of *Phlebotomus argentipes* blood-sucking flies, reported previously (*22*). This species seems to breed essentially in cattle sheds (*23*) and are 5× more attracted to cattle than to persons (*24*,*25*) and feed more on animals (62.80%) than on persons (24.92%), according to a study in India (*22*).

Information about the association of PCR results for persons and domestic animals can be used to investigate the role of domestic animals in *L. donovani* transmission. Visual inspection of the data suggested that most of the *Leishmania* spp.–positive persons were living near *Leishmania* spp.–positive goats. This observation was confirmed by bivariate K-function results and CT analysis. Distance of clustering between *Leishmania* spp.–positive persons and *Leishmania* spp.–positive domestic animals varied slightly according to the method (K-function up to 5 m; CT analysis <22.8 m) and was less than the flight range of *P. argentipes* flies. Differences could be explained by the inclusion of *Leishmania* spp.–negative households in the CT analysis. The 2 types of CT analyses pointed more to the role of biologic factors than to household factors like education, bednet use, or type of house. Additional studies should explore the presence of poultry as a risk factor, as has been reported for urban VL in Brazil (*26*).

Even if our results indicate that goats might be involved in the dynamics of VL, they do not necessarily mean that these animals constitute a reservoir host for *L. donovani*. Criteria for the definition of *Leishmania* reservoir hosts were recently reviewed (*27*) and include sandfly foraging behavior and feeding preferences and the dynamics of infections in assumed reservoir hosts; a key question is the clearance times (chronicity) of infections. Whether the phenomenon observed here can be extrapolated to other VL-endemic foci should also be explored. Dharan-17 is a new emerging focus, and in the absence of immunity, human and animal populations could be more sensitive to *Leishmania* infections. Our observations warrant further investigation and a close monitoring of goats and other peridomiciliary animals like rodents and birds. If the role of these animals in the transmission cycle is confirmed, the potential implications could affect VL control programs in the region.

Acknowledgments

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Investigating an Airborne Tularemia Outbreak, Germany

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In November 2005, an outbreak of tularemia occurred among 39 participants in a hare hunt in Hesse, Germany. Previously reported tularemia outbreaks in Germany dated back to the 1950s. We conducted a retrospective cohort study among participants and investigated the environment to identify risk factors for infection. Ten participants had serologic evidence of acute *Francisella tularensis* infection; 1 other participant died before laboratory confirmation was obtained. Presence within 5 meters of the place where disemboweled hares were rinsed with a water hose was the risk factor most strongly associated with infection (risk ratio 22.1; 95% confidence interval 13.2–154.3). Swabs taken at the game chamber and water samples were PCR negative for *F. tularensis*. Eleven of 14 hare parts showed low-level concentrations of *F. tularensis*, compatible with cross-contamination. More than half of case-patients may have acquired infection through inhalation of aerosolized droplets containing *F. tularensis* generated during rinsing of infected hares.

In the last 50 years, few laboratory-confirmed outbreaks of airborne tularemia have been described. They include outbreaks in workers in sugar cane factories in Ukraine, the Czech Republic, and Austria (*1*–*3*); farmers in Sweden and Finland (*4*,*5*); and residents of the island of Martha's Vineyard, Massachusetts, USA (*6*). Small clusters and outbreaks with probable common source exposure may have been associated with disturbance of contaminated animal carcasses (*7*–*9*) and dogs with contaminated fur shaking themselves inside houses (*10*,*11*). In Germany, tularemia is rare, with only 184 cases reported during 1955–2004 (*12*). Starting in late 2004, tularemia reemerged, causing repeated outbreaks in nonhuman primates at different animal facilities in central Germany (*13*), followed by rising numbers of human cases in 2005, 2007, and 2008. Here we report a point-source outbreak of tularemia among participants of a hare hunt in Hesse, Germany, in November 2005.

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The Outbreak

On December 1, 2005, Darmstadt health authorities were notified of a laboratory-diagnosed case of tularemia. The patient had participated in a hare (*Lepus europaeus*) hunt on October 29, 2005, and cut 1 finger while disemboweling and skinning hares. On November 2, the patient became ill with fever >40°C, axillary lymphadenopathy, arthralgia, and headache. Initially treated as an outpatient, he was hospitalized November 21 for progressive lymphadenitis and recurrent fever; *Francisella tularensis* infection was diagnosed by lymph node biopsy and specific antibodies. After the Darmstadt-Dieburg Public Health Authority received notification of this index case, that agency initiated an outbreak investigation.

On October 29, 2005, 29 hunters and 10 beaters, who drove hares out of areas of cover, participated in the hunt. Sixty-three hares were shot. Some hares were disemboweled where they were shot; most were later disemboweled and rinsed with a water hose at a hunting lodge. Disemboweled hares were transported to a game chamber and skinned the next day.

Materials and Methods

Patients

All participants of the hunt were offered serologic testing. From December 3, 2005, through March 3, 2006,

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serum was obtained from 29 participants, and DNA was extracted from an affected lymph node of the index case-patient.

Two different case definitions were used. Symptomatic participants of the hunt who fell ill during October 30–November 12, 2005, were defined as confirmed case-patients if they had a single high titer of *F. tularensis*–specific antibodies. We defined a probable case-patient as either an asymptomatic hunt participant with a single high titer of *F. tularensis*–specific antibodies or a hunt participant who had signs and symptoms suggestive of *F. tularensis* infection from October 30 through November 12, 2005, but no laboratory confirmation.

Retrospective Cohort Study

Starting December 13, 2005, we interviewed hare hunt participants using a standardized questionnaire to determine demographic and clinical details and risk factors for *F. tularensis* infection. For statistical analysis, we combined probable and confirmed cases; all participants who did not fulfill a case definition were included as controls. All analyses were performed with Intercooled STATA 10.0 for Windows statistical software (StataCorp, College Station, TX, USA). Fisher exact test was used to analyze the relationship between categorical variables and the 2-sample Wilcoxon rank-sum test used to analyze the relationship between numeric data and the categorical outcome.

Environmental Investigation

Starting in early December 2005, we visited the outbreak area 3 times. We obtained data on elevation, regional mean annual air temperature, precipitation, and sunshine hours (1961–2004) from the Federal Meteorological Service (Offenbach am Main, Germany). Water samples were obtained from a small creek near the hunting lodge and from the water hose used to rinse disemboweled hares. Additionally, 28 samples were taken at the game chamber (Table 1; Figure 1). All samples were stored at 4°C.

Deep frozen parts from 12–14 hares shot during the initial hunt on October 29 were recovered from different households. Additional animals were shot in the same hunting area on December 6, 2005, and January 7 and 14, 2006. In January 2006, all frozen samples were transported on dry ice to a microbiologic laboratory.

Laboratory Methods

Direct Detection of *F. tularensis*

All animal samples were stored at –20° C until preparation for PCR, antigen detection, or culture. Specimens of spleens, livers, bone marrow, and muscle tissue of the animals were homogenized as described recently (*13*) and tested for *F. tularensis*–specific lipopolysaccharide (LPS) using a capture ELISA (*14*) or an immunochromatographic column assay (ABICAP, Senova, Jena, Germany). Purified DNA was prepared from tissue homogenates, blood, and water samples and from fluids obtained during thawing of the hare samples by using the QIAamp Tissue kit (QIAGEN, Hilden, Germany).

PCR amplification and product detection were performed in a LightCycler instrument (Roche, Mannheim, Germany) by using a commercially available real-time PCR kit (TibMolBiol, Berlin, Germany) for the detection of a specific nucleotide sequence within the 16S rRNA gene of *F. tularensis* (*15*). Additionally, real-time PCR protocols targeting the *tul4* (*16*), *iglC*, *ISFtu2*, or *fopA* gene were performed (*17*). Each run included positive and negative controls. For subspecies identification, a conventional PCR protocol employing primers flanking the RD1 region of *F. tularensis* was used (*18*). To prove the presence of *F. tu-*

Table 1. Type of environmental samples taken and results of testing for *Francisella tularensis*, Germany, 2005*

Type	No. samples	Origin	Results		
			PCR	Culture	Ag detection (LPS)
Water	2	Creek, water hose	Neg	Neg	Neg
Swabs	16	Game chamber	Neg	Neg (4/4)	–
Hare fur, insects	3	Game chamber	Neg	–	–
Liquid samples (flush)	9	Game chamber	Neg	–	-
Frozen parts of hares received from 9 different households (muscle, bone marrow, fluids recovered during thawing)	14	12–14 hares (*Lepus europaeus*), shot 2005 Oct 29	Pos 11*/14	Neg (6/6)	Pos. 6†/14
Liver/spleen samples	29	15 hares, 1 nutria (*Myocastor coypus*), shot 2005 Dec 12	Neg	–	–
Organs of hares (liver, spleen, whole blood)	72	24 hares, shot 2006 Jan 7 and Jan 14	Neg‡	–	–

*For all parts, tissue, bone marrow, and fluid from thawing were tested. Samples were considered positive when ≥2 materials were repeatedly positive by 2 different PCRs. Ag, antigen; LPS, lipopolysaccharide; Neg, negative; Pos, positive.
†Samples also positive by PCR.
‡PCR inhibition noticed for 19/24 blood samples.

Figure 1. Sampling at the game chamber, Hesse, Germany, December 2005.

larensis–specific DNA in hares showing a low signal in the screening PCR, we performed amplification of a 16S rRNA gene target followed by sequencing of the fragment.

Serum Samples and Culture Recovery of *F. tularensis*

Water samples; swab samples; and spleen, liver, and bone marrow homogenates were cultured on cysteine heart agar supplemented with 9% sheep blood, Columbia blood agar, McConkey agar, and modified Thayer-Martin medium containing antimicrobial drugs (Merck, Darmstadt, Germany). Culture plates were incubated at 37°C for 10 days and investigated daily for bacterial growth (*13*). Serum from 29 participants was examined for *F. tularensis*–specific anti-LPS antibodies by a qualitative screening ELISA and confirmed by immunoblot (*18*).

Results

Patients: Clinical Characteristics and Laboratory Results

Characteristics of 9 hare hunt participants met the definition of a confirmed case; 2 participants had characteristics that met the definition of a probable case. The median age of case-patients was 55 years (range 11–73 years); all were male. Illness onsets ranged from November 2 through November 7 (Figure 2). One probable case-patient (no. 3 in Figure 2) fell ill with high fever, myalgia, and clinically diagnosed bilateral pneumonia; he had chronic heart failure and died during the second week of illness despite treatment with moxifloxacin. Neither specific antibodies nor *F. tularensis*–specific DNA could be detected in a serum specimen taken 8 days after illness onset. The second probable case-patient was asymptomatic but had high levels of anti-LPS–specific antibodies (immunoglobulin [Ig] M 32,000; IgA 32,000; IgG 8,000), suggesting a recent subclinical infection. Antibody titers of the 9 confirmed case-patients ranged from 64,000 to >256,000 (negative ≤500). All 9 showed a specific IgG, IgA, and IgM immune response, all were medically attended, and 1 was hospitalized. They reported fever ≥38.5°C (range 38.5°C–40.6°C) (8 persons), chills (6), headache (5), weight loss (5), myalgia (5), enlarged lymph nodes (3), and coughing (1). None reported sore throat or pneumonia. Two case-patients had an ulceroglandular form of tularemia: the index patient (case-patient 1 in Figure 2) had cut his finger while skinning hares; the other (case-patient 6) had scratched his finger before the hunt.

PCR of an affected lymph node specimen and sequencing of the amplificate indicated *Francisella* infection. Real-time PCR (targets 16S rRNA gene, *tul4* gene) confirmed the presence of *F. tularensis*–specific DNA within the sample. Partial amplification of the RD 1 region identified a 923-bp fragment considered to be specific for subspecies *holarctica* (*18*). Several attempts to amplify *F. tularensis* DNA fragments from serum of case-patient 3 were unsuccessful.

Retrospective Cohort Study

The analysis included data for 10 of the 11 case-patients and all 28 controls (Table 2). Presence within 5 meters of where disemboweled hares were rinsed was the risk factor most strongly associated with infection. Case-patient 3, who died, was not included in the cohort study; however, he was reported to have disemboweled hares within 5 meters of the area where disemboweled hares were rinsed. Hares were disemboweled and rinsed at the hunting lodge during the lunch break and in the afternoon after the hunt. Ten case-patients were at the lodge at the end of the hunt; 7 were at the lodge during the lunch break. In case-patient 6, who was not at the hunting lodge in the afternoon, ulceroglandular tularemia developed. The asymptomatic hunter (no. 11) had disemboweled ≈12 hares at a distance 8–10 meters from the place where hares were rinsed. One person present at the hunting lodge, whose laboratory tests were negative for *F. tularensis*, reported that although he

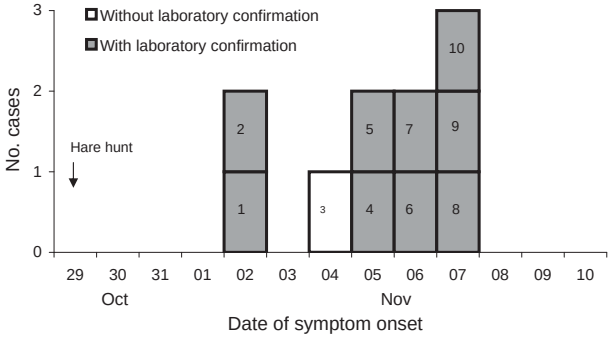

Figure 2. Tularemia cases (n = 10), by symptom onset, County of Darmstadt-Dieburg, Germany, October–November 2005.

Table 2. Attack rates among exposed and nonexposed hare hunters, according to potential risk factors for *Francisella tularensis* infection, Germany, 2005*

Potential risk factor	Exposed			Not exposed			RR (95% CI)	p value
	No. cases	No. hunters	Attack rate, %	No. cases	No. hunters	Attack rate, %		
Hunted	8	27	29.6	2	10	20.0	1.5 (0.4–5.8)	0.45
Had direct contact with dead hares	10	34	29.4	0	4	0	–	0.2
Wore gloves during contact with dead hares	2	5	40.0	8	21	38.1	1.5 (0.4–4.9)	0.6
Injured skin	4	8	50.0	5	25	20.0	2.5 (0.9–7.1)	0.12
Disemboweled hares	7	11	63.6	3	27	11.1	5.7 (1.8–18.2)	0.002
Injured while disemboweling hares	1	2	50.0	9	36	25.0	2.0 (0.5–8.9)	0.46
Rinsed hares at the lodge	4	6	66.7	6	32	18.8	3.6 (1.4–8.9)	0.03
Presence within 5 m of where disemboweled hares were rinsed	9	11	81.8	1	27	3.7	22.1 (3.2–154.3)	<0.0001
Skinned hares	5	9	55.6	5	29	17.2	3.2 (1.2–0.7)	0.04
Injured while skinning hares	1	1	100	9	37	24.3	4.1 (2.3–7.3)	0.26
Had contact with raised dust	4	5	80.8	6	33	18.2	4.4 (1.9–10.3)	0.01
Had contact with puddle or ditch water	0	1	0	10	37	27.0	–	0.74
Received tick bite on the hunting day	0	0	–	10	38	26.3	–	–
Consumed hares hunted on October 29, 2005	0	4	0.0	10	34	29.4	–	0.28
Attended the common lunch/supper	9	35	25.7	1	3	33.3	0.8 (0.0–4.2)	0.61
Stayed abroad in October 2005	4	7	57.1	6	30	20.0	2.9 (1.1–7.5)	0.07

*RR, risk ratio; CI, confidence interval. Totals vary because of answers of "do not know."

had been within 5 meters of the area where disemboweled hares were rinsed, he preferred to keep some distance from the group that were handling the hares.

Environmental Investigations

The outbreak area has several ecologic characteristics that were shown to correlate with high numbers of tularemia foci in the Czech Republic (Table 3). According to the hunters, all hares shot during the hunt on October 29 appeared healthy and showed no macroscopic signs of systemic infection (e.g., enlarged liver or spleen). No die-off of hares or rodents was observed in the region.

Samples taken in the game chamber and of the water were negative for *F. tularensis*, whereas samples taken from 11 of 14 parts of hares from the initial hunt were positive (Table 1). Six of these samples were additionally positive for *F. tularensis*–specific LPS.

Discussion

We investigated an outbreak of tularemia after a hare hunt in Hesse, Germany, for which epidemiologic, clinical, and microbiologic data indicate an airborne origin. The results of the cohort study support this hypothesis on the basis of the association between case status and presence within 5 meters of the location where disemboweled hares were rinsed. During the afternoon session of disemboweling and rinsing hares, 10 of the 11 case-patients were at the hunting lodge; aerosolization of infectious particles may have been limited to this session. Three case-patients, among them the patient who did not participate in the afternoon session, had a glandular or ulceroglandular form of tularemia. They may have acquired infections through skin lesions. The absence of cutaneous lesions or lymphadenopathy in the remaining 8 patients makes a cutaneous route of infection less likely than a respiratory route. The low incidence of respiratory symptoms among our patients is in agreement with findings from previous airborne outbreaks that involved patients infected with the less virulent subspecies *F. tularensis holarctica*, in which only a minority of case-patients had symptoms suggestive of pneumonia (*8*,*9*).

Two hunters met the probable case-patient definition. The asymptomatic hunter (no. 11) disemboweled hares at a distance from the group. Severity of clinical tularemia has been correlated with infectious dose (*20*), and this hunter

Table 3. Ecologic characteristics of the outbreak area in Germany and of natural habitats correlated with a high number of tularemia foci in the Czech Republic

Characteristic	Outbreak area, Germany, 2005	Natural habitats in the Czech Republic with high numbers of tularemia foci (*19*)
Elevation above sea level	88–112 m	Up to 200 m
Mean annual air temperature	10.0°C (1994–2004)*	8.1–10.00°C
Mean annual precipitation	673.5 mm (1994–2004)*	450–700 mm
Habitat	Single trees along a creek, alluvial forest-like field biotope, surrounded by areas of intensive agriculture	Alluvial forests, field biotopes
Mean annual sunshine duration	1,685 h (1994–2004)*	2,001–2,200 h

*Ten-year period preceding the year in which the outbreak occurred.

might have been exposed to a smaller pathogen load or exposed on another recent occasion. Case-patient 3 died during the second week of illness. Antibodies against *F. tularensis* in most patients appear 6–10 days after onset of symptoms (*21*,*22*). Serum available for testing from case-patient 3 was from his eighth day of illness; hence, it was possibly taken before a measurable antibody response developed. We further cannot exclude the possibility that the 10 asymptomatic participants who did not undergo laboratory testing had to be considered as probable case-patients if they provided a serum sample.

Detection of *F. tularensis* in hare specimens, including bone marrow specimens, and lack of *F. tularensis* detection in samples of the water system used to rinse hares suggest infection of the hares. One or more infected hares, still bloody and wet, may have cross-contaminated additional hares during further processing, e.g., during transport to and storage at the game chamber. All samples taken in the game chamber showed negative results. Case-patient 3 had cleaned the game chamber thoroughly with a pressure washer, possibly exposing himself to a high pathogen load.

Small clusters and outbreaks of airborne tularemia have been associated with hares or rabbits (*7*–*11*). However, most cases of tularemia associated with hares are of the ulceroglandular or glandular form (*1*,*22*). In a protracted outbreak in Spain, 97% of patients reported previous contact with hares; 71% of these had a glandular or ulceroglandular form of disease (*23*). Of 577 case-patients treated at a clinic in Czechoslovakia, 194 had direct contact with hares, and an (ulcero) glandular form of disease developed (*1*). Different frequencies of the diverse clinical forms of tularemia suggest differences in the main route of pathogen acquisition.

In the retrospective cohort study, presence within 5 meters of the place where disemboweled hares were rinsed was the risk factor most strongly associated with infection. Washing of contaminated produce was found to be an effective mechanism of generating infectious aerosols in tularemia outbreaks in sugar beet factories (*1*–*3*), and rinsing ≥1 hares infected with *F. tularensis* was the most probable way by which an infectious aerosol was generated. However, we cannot exclude the idea that an infectious aerosol was formed through further hare manipulating activities, e.g., transport.

Previous outbreaks in Germany date back to the 1950s, with the last case reported in the outbreak area in 1957 (*24*). Environmental characteristics of natural foci of tularemia persisting over >30 years have been described (*19*,*25*). The outbreak region in Germany shares several features favoring the occurrence or persistence of *F. tularensis* in the environment. Recently, the presence of *F. tularensis* in trapped rodents (2.1%) and in water samples from this hunting area was directly confirmed, and >10% of rodents in several German regions not previously considered as endemic foci were infected (*19*). In addition, *F. tularensis* was repeatedly detected in 22 hares from 5 federal states (Bavaria, Hesse, Baden-Wuerttemberg, Thuringia, and Lower Saxony) after improved diagnostic tools (real-time PCR) had been applied complementary to standard 48-h bacterial cultivation (W.D. Splettstoesser et al., unpub. data). Together with results obtained from serologic studies in the German population (*26*), the outbreak reported here suggests that tularemia has either reemerged in Germany or is seriously underreported.

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Statewide School-located Influenza Vaccination Program for Children 5–13 Years of Age, Hawaii, USA

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New guidance recommends annual influenza vaccination for all children 5–18 years of age in the United States. During 2007–2008, Hawaii offered inactivated and live attenuated influenza vaccine at school-located clinics for grades kindergarten through 8. Most (90%) public and private schools participated, and 622 clinics were conducted at 340 schools. Of 132,775 children 5–13 years of age, 60,760 (46%) were vaccinated. The proportion vaccinated peaked at 54% for those 6 years of age and declined for older cohorts. More than 90% of schoolchildren transited the clinic in <10 minutes. A total of 16,920 staff-hours were expended; estimated cost per dose administered was $27 and included vaccine purchase and administration, health staffing resources, printing costs, data management, and promotion. This program demonstrates the feasibility of conducting mass school-located influenza vaccination programs in public and private schools statewide, as might be indicated to respond to pandemic influenza.

Seasonal influenza reportedly results in 200,000 hospitalizations and 36,000 deaths annually in the United States (*1*). In addition, ≈31 million outpatient visits are attributable to influenza during seasonal epidemics; annual projected direct medical costs are $10.4 billion and lost earnings are $16.3 billion (*2*). Although schoolchildren are not considered at high risk of dying from influenza, annual illness attack rates in schoolchildren were >40% in some years (*3*,*4*). In addition, children 5–18 years of age may be the primary source of communitywide influenza transmission (*5*–*7*). Focused prevention of influenza infection among children may not only prevent childhood illness but also decrease school absenteeism and reduce the negative impact of influenza infection among working adults and elderly persons (*8*–*10*). Disease modeling suggests that if influenza vaccine was limited, as might be expected during a pandemic, vaccinating schoolchildren might be the most efficient approach to reducing overall numbers of influenza infections (*11*–*14*).

On February 27, 2008, the Advisory Committee on Immunization Practices (ACIP) expanded the recommended ages for annual influenza vaccination of children to include all children 6 months–18 years of age (*15*). Nationwide, this new recommendation added ≈30 million children to the cohorts targeted for annual influenza vaccination and invoked calls to consider alternatives to the physician's office for administering the vaccine (*16*,*17*).

School-located influenza vaccination (SLIV) clinics have been proposed as a way to get more children vaccinated. Results from several trial SLIV programs indicate that vaccinated schoolchildren and their families experience lower rates of influenza-associated illnesses (*18*–*20*). However, many recent studies used only live attenuated influenza vaccine (LAIV), administered as a nasal spray, donated by the manufacturer (*6*,*21*). LAIV is currently not recommended for use in children with asthma or other underlying medical conditions that predispose them to complications from wild-type influenza infection; yet these potentially high-risk children may derive the most direct benefit from vaccination against influenza.

As a result, during the 2006–07 school year, the State of Hawaii Department of Health (DOH) conducted a pilot project to assess the feasibility of providing a choice of intranasal LAIV or intramuscular trivalent influenza vaccine (TIV) to students at 3 elementary schools. The pilot project achieved an overall vaccination rate of 35%. On the basis

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of this success, DOH conducted a statewide SLIV program during the 2007–08 influenza season. We present data on the logistics and outcomes of implementing this large-scale public health program; these data may be relevant to jurisdictions planning annual seasonal influenza vaccination programs for children and for responding to recent ACIP recommendations that place school-aged children among the top priority groups for receiving Influenza A (H1N1) 2009 Monovalent Vaccine (*22*,*23*).

Methods

The 2007–08 SLIV program focused on all children 5–13 years of age in Hawaii. Public and private elementary and middle schools were identified through the databases of the Hawaii Department of Education, Hawaii Association of Independent Schools, and Hawaii Catholic Schools. All schools were invited to participate and could register online.

Influenza vaccination was voluntary and required written consent from the child's parent or guardian. Information packets contained an explanatory letter, the 2007–08 Influenza Vaccine Information Statements for both vaccine formulations, and a consent form. A parent or guardian could specifically consent to their child receiving TIV or LAIV or indicate that either formulation was acceptable. To guide the parental decision, the consent form asked about potential contraindications to each formulation. If the child was <9 years of age and had not been vaccinated with 2 doses of influenza vaccine in any previous year (or if the parent, medical provider, or both were uncertain), parents were asked to consent to 2 doses of influenza vaccine administered ≥6 weeks apart. The packets were distributed to children at participating schools in August 2008; the consent forms were collected by the schools ≈4 weeks later. The packets were available online in 11 languages other than English commonly spoken in Hawaii (www.stopfluatschool.com). Families incurred no cost and received no incentive for participation. DOH provided the vaccine and all clinic supplies. School-based faculty and staff also were offered influenza vaccination at no cost through SLIV clinics.

Vaccines were administered at school during normal school hours over 59 working days from October 15, 2007, through January 31, 2008. School clinic dates and times were established through dialogue with the principal or other administrator of each school. School administrators at participating schools were asked to provide a large room for up to 4 hours (two 4-hour sessions >6 weeks apart for elementary schools), notify parents of the date and time of the scheduled vaccination clinic, and escort students to and from clinics. DOH staff, assisted by contract courier services, transported clinic supplies from DOH offices to each school 1 day before the clinic. DOH paid for all vaccine from state and federal funds and arranged all clinic staffing. No money was billed to third-party payers or collected on site.

Each school clinic required 1 clinic manager and ≥1 registration personnel and vaccinators, according to the number of vaccinations anticipated there. Staffing data (position, affiliation, hours spent) were determined prospectively and available for 92% of all clinics. For the remainder, we imputed their values from the mean value of the clinics with data available.

Participating DOH personnel, volunteers, and contract staff received DOH-developed training tailored to their respective program responsibilities. DOH verified that all vaccinators were licensed health professionals in good standing; the exception was nursing school students who worked under the on-site supervision of licensed faculty preceptors. In accordance with Hawaii state law, volunteers for DOH, including Medical Reserve Corps volunteers, were considered state employees for liability purposes.

We defined student age in years as date of first influenza vaccination administered by this program minus the child's date of birth divided by 365.25. Student population size for the cohort of children aged 5–13 years residing in Hawaii on July 1, 2007, was obtained using publicly available census estimates (*24*).

To calculate school-level participation rates, the number of children enrolled in grades kindergarten through 8 (K–8) at individual schools was obtained from the Hawaii Council of Private Schools, Private School Enrollment Report 2007–2008, and the State of Hawaii Department of Education Official 2007–08 Enrollment. To calculate the proportion of school staff vaccinated, we obtained the total number of personnel employed at each school from the participating schools.

To calculate clinic throughput times, a nonrandomized subset of children were given a card documenting the time they arrived at the registration area. The card was collected and exit time noted as the child left the clinic area after vaccination. The difference between exit and arrival times, rounded to the nearest minute, was the clinic throughput time. Although we did not randomly select these students, we tried to obtain a representative sample by distributing the time-stamped cards to 10 children spread over the operational period at each of nearly 200 clinics, totaling ≈3% of vaccinated students.

We collected reports of potential adverse events after vaccination sent to the national passive surveillance system (Vaccine Adverse Event Reporting System) (*25*). We also maintained records of calls DOH received directly from parents, clinicians, or schools regarding potential adverse events.

Costs were estimated from the DOH perspective so that in-kind contributions from schools whose staff disseminated

or collected consent materials and/or escorted students to clinics were not included. We estimated the cost of in-kind contributions of DOH staff and volunteers using 2007 salary data available from the US Department of Labor Bureau of Labor Statistics (*26*). Clinic manager hourly costs were defined as the national hourly wage for registered nurses at the 75th percentile ($35.18); vaccine administrator hourly costs were defined as the median hourly wage for registered nurses ($30.44), and registration personnel hourly costs were defined as the median hourly wage for medical records and health information technicians ($14.08).

We used Epi Info 2000 to calculate means and standard deviations for computing 95% confidence intervals (CIs); Epi Info version 6 χ^2 test for trend was used to assess trends in proportions over successive age cohorts (*27*). Correlation coefficients were calculated by using Excel (Microsoft, Redmond, WA, USA).

Results

Of 67,203 schoolchildren for whom consent was obtained, 63,153 (94%) received ≥1 doses of influenza vaccine. Of schoolchildren receiving at least 1 influenza vaccination, 1,078 (1.7%) were <5 years of age, 60,760 (96%) were 5–13 years of age, and 1,136 (1.8%) were >13 years of age; age information was missing for 179 (0.3%) children. Thus, 46% of 5–13-year-old children in Hawaii, as determined by census data (n = 132,775), were vaccinated against influenza at SLIV clinics.

Twenty-nine percent (n = 18,173) of children who received a first influenza vaccination at an SLIV clinic received a second dose, representing 60% of the 30,357 children <9 years of age who participated in the program. A total of 81,326 first or second vaccine doses were provided to children at SLIV clinics.

In addition, 9,306 (43%) of the 21,625 school staff members and 1,054 clinic volunteers were vaccinated at SLIV clinics, bringing the overall total number of influenza vaccinations administered through the program to 91,686. These 2 groups represented 14% of vaccinated persons.

A total of 340 (90%) of 377 elementary and middle schools in Hawaii participated in the program. This number constitutes 242 (96%) of 251 public schools and 98 (78%) of 126 private schools.

A total of 622 SLIV clinics were conducted; 345 (55%) were first-dose clinics during October through December 2007; 5 large schools had 2 first-dose clinics because of the number of vaccinees anticipated. Another 277 (45%) were second-dose clinics conducted at elementary schools in January 2008 to fully vaccinate children <9 years of age who had never received 2 doses of influenza vaccine in a prior year.

Unless indicated otherwise, the following analyses are restricted to children 5–13 years of age. In Honolulu County, 48% of schoolchildren were vaccinated; in Hawaii's other counties, 39%–42% were vaccinated (Figure 1).

Vaccinations peaked at 54% for children 6 years of age. In successive age cohorts, the vaccination rates gradually declined to 30% for children 13 years of age (Figure 2).

Grade-level enrollment data required for calculation of school-level participation rates were available for 291 (86%) of the 340 participating schools. The proportion of children vaccinated at individual schools ranged from 3% to 84% (Figure 3). However, the mean proportion of schoolchildren vaccinated was similar between the 208 public (43.4%; 95% CI 42.1%–44.7%) and 83 private (45.0%; 95% CI 41.5%–48.5%) schools. School size, as measured by the number of students in grades K–8, correlated poorly with vaccination coverage rates obtained at the school (correlation coefficient: –0.05, Figure 4).

Most (56%) parents selected TIV for their child; 27% chose LAIV; and 17% consented to have their child receive either formulation (p<0.001). Vaccine formulation preference did not differ significantly by student sex (p = 0.19). However, the trend for parents to select TIV (only) over LAIV or either vaccine in successive annual age cohorts was significant (p<0.001, Figure 5).

Clinic throughput times were obtained for 1,970 schoolchildren vaccinated at 199 separate clinics. Median throughput time was 4 minutes; >90% of schoolchildren transited the clinic in ≤10 minutes.

Physicians submitted vaccine adverse event reports for 3 children who received TIV through the school program; no events were medically serious or required hospitalization. In addition, DOH staff were informed of 4 other minor incidents, of which 3 were vasovagal syncopal episodes after TIV administration.

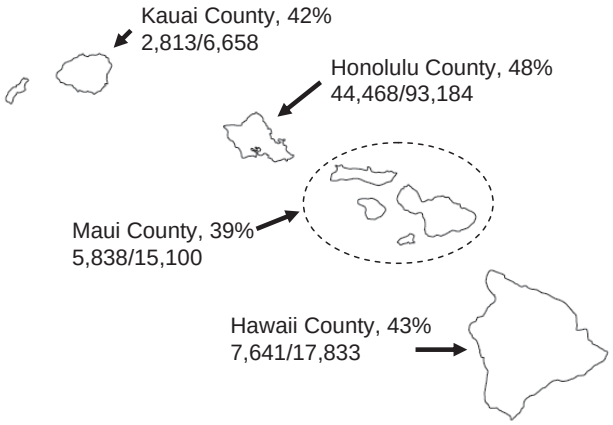

Figure 1. Number and proportion of children 5–13 years of age receiving ≥1 doses of influenza vaccine at school-located clinics, by county, Hawaii, USA, 2007–08 influenza season. Numerator is the number of children 5–13 years of age vaccinated in the program; denominator is the county population of children 5–13 years of age as of July 1, 2007.

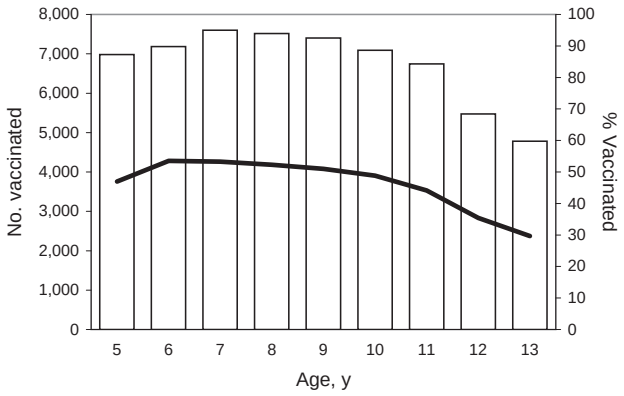

Figure 2. Number and percentage of children 5–13 years of age receiving at least 1 dose of influenza vaccine through a school-located clinic, by year of age, Hawaii, USA, 2007–08 influenza season. White bars indicate number of children vaccinated; black line indicates percentage of children vaccinated.

A total of 16,920 person-hours were expended to conduct 345 first-dose clinics and 277 second-dose clinics (Table 1). Mean duration of the first-dose clinic was 3.1 hours and typically required 1 clinic manager, 5 or 6 registration staff members, and 6 vaccinators. Second-dose clinics averaged 2.3 hours and used 2 registration staff members and 2 or 3 vaccinators**.**

DOH public health nurses accounted for 80% of clinic manager person-hours; other DOH staff provided almost 75% of person-hours for registration (Table 1). Contract nurses accounted for almost 50% of all vaccinator staff hours; DOH public health nurses and nursing school students filled most of the remaining need. Other organizations providing substantial staff support included the Hawaii Medical Reserve Corps and the US Department of Defense**.**

Program operation costs were estimated to be $2,480,493; nearly half was used to purchase vaccine. Forty-six percent of vaccine doses were acquired through the federal Vaccines for Children program (Table 2). The all-inclusive cost of administering 90,632 doses of influenza vaccine to participating children and school staff, comprising vaccine purchase and administration, healthcare staff resources, printing costs, data management, media promotion, and schoolchild participation rewards, was $27.37 per dose.

Discussion

This is the largest reported school-located influenza vaccination program in the United States and the only one to offer parents a choice of influenza vaccine formulation, i.e., TIV or LAIV (*28–31*). Through this effort, we vaccinated nearly half of all children 5–13 years of age in Hawaii against influenza.

Several valuable lessons can be learned. School vaccine coverage rates varied widely throughout the state, regardless of school size or school type (i.e., public vs. private), suggesting that other factors were important in determining acceptance of mass influenza vaccination at school. More work is needed to examine socioeconomic, cultural, and school-level characteristics that might influence participation in school-based vaccination programs.

A large proportion of public and private schools participated in the SLIV program. DOH promoted the program to school principals as a means to reduce influenza illness among students and staff, potentially resulting in decreased influenza-associated absenteeism. The high participation rates suggest that many principals believed the investment of student and staff time for the SLIV clinics was acceptable.

Vaccination rates declined among older, middle school–aged cohorts, suggesting that obtaining high influenza vaccination rates among high school-aged students may be challenging. This observation is consistent with low influenza vaccination rates attained at a high school in Hawaii during the 2006–07 school year and with our experience with school-located hepatitis B vaccination programs in Hawaii during the 1990s (Hawaii DOH, unpub. data).

The second-dose clinics were resource intensive. These clinics accounted for 45% of clinic sessions but for only 22% of vaccine administered. If the 2006 ACIP recommendation to vaccinate all children beginning at 6 months becomes widely implemented, successively fewer school-aged children would require a second dose of vaccine, potentially obviating the need for second-dose SLIV clinics.

When offered a choice, parents selected the injectable vaccine for their children over the nasally administered attenuated formulation by a 2-to-1 margin. Although some parents would have based their choices on differences in contraindications to the 2 vaccines, the magnitude of this difference and the increasing preference for TIV for older

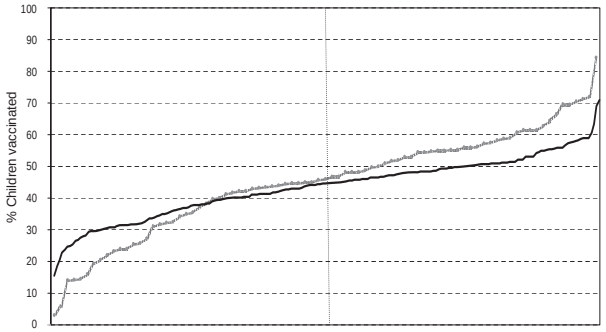

Figure 3. Vaccination rate ranking, by school (grades K–8), public and private schools, Hawaii, USA, 2007–08 influenza season. Black line indicates public schools ranked 1–208 (left to right); gray line indicates private schools ranked 1–83 (left to right). Vertical line indicates the median.

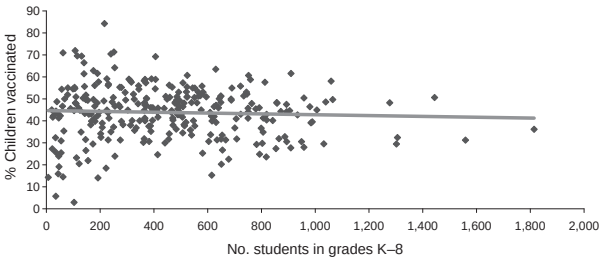

Figure 4. Proportion of children enrolled in grades K–8 at each school who received at least 1 dose of influenza vaccine, by school size, Hawaii, USA, 2007–08 influenza season. Linear fit trend (gray line) calculated by using Excel software (Microsoft, Redmond, WA, USA); r = –0.05.

children suggests that many parents simply preferred the injectable formulation. We did not investigate the reasons but speculate that parents were more familiar with TIV. Anecdotal reports from clinics indicated that some children sought the injectable vaccine to demonstrate their machismo to their peers, and several children are known to have switched from the LAIV to the TIV waiting line, despite their parents' wishes. Ongoing assessment of these attitudes and behaviors are important to determine whether the preference for injectable vaccine persists as parents become more familiar with nasally administered vaccine.

Despite substantial program promotion and favorable media attention, we did not reach our informal target of 50% coverage. Achieving higher rates may require changes in parental perceptions about the risk for influenza illness in children, with the potential benefits of vaccination outweighing any perceived potential risks to the child. Still, almost 30% of schools vaccinated more than half of their students in grades K–8 before the 2008 ACIP expanded recommendation (*1*). The new recommendation for school-aged children may facilitate achieving higher vaccination coverage in future years.

Each year, <30% of children 5–13 years of age are estimated to see a medical provider at any time during October through December, i.e., the usual time frame for administering influenza vaccine (*32*). Applying this figure to the population of children vaccinated in our SLIV program, we find that ≈42,532 additional visits to a physician's office would be needed to reach the same level of coverage. Whether sufficient capacity exists to add these additional office visits to providers' schedules can be debated; what seems clear, however, is that the SLIV clinics remove potentially substantial barriers to vaccination, i.e., the need for parents to secure transportation and take time away from work or other duties to bring their child into the provider's office. SLIV clinics are convenient for parents and do not generate indirect costs through lost wages. Research among adults indicates that the mean cost of delivering influenza vaccinations is lower when they are provided in mass vaccination settings compared with scheduled office visits (*33*). More study is needed to comprehensively assess the costs and benefits of providing influenza vaccines to children at SLIV clinics.

Our study had at least 1 major potential limitation. Because the State of Hawaii has a relatively small population, one might question the generalizability of our findings to larger jurisdictions on the US mainland. However, with a population >900,000, Honolulu County has more residents than 98% of other US counties and one of the nation's highest population densities (*34*). The SLIV program in Honolulu County generated a vaccination coverage rate comparable with that of the other 3 less populous counties, each with 60,000–172,000 residents. The consistency of our experience across large and small counties suggests that our results may be applicable to other jurisdictions considering implementation of an SLIV program.

This program demonstrates the feasibility of large-scale SLIV programs among children in elementary and middle schools offering both TIV and LAIV. No medically serious vaccine-associated adverse events were identified. Anecdotal information indicated that the program was well received; many parents and school staff requested that we repeat the SLIV clinics the following year. In response, the statewide program was continued for the 2008–09 school year and achieved similar rates of vaccination coverage for the 5–13-year-old cohort, as well as school participation rates, compared with the inaugural year (45% and 89%, respectively). The logistical effort required to conduct this program greatly improved our organizational capacity to conduct sustained mass vaccination clinics for children as might be required in response to a pandemic (*22*,*23*). In addition, this program

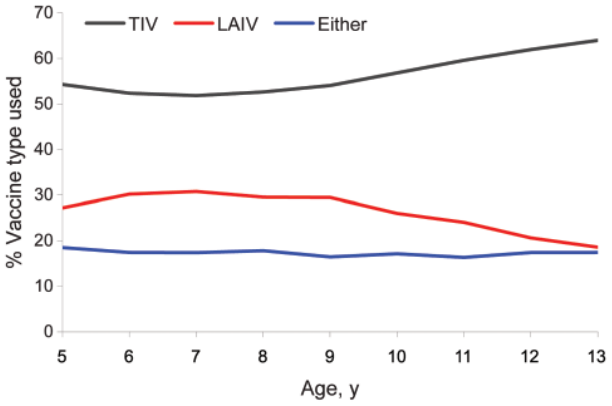

Figure 5. Proportion of children receiving at least 1 dose of influenza vaccine in school-located clinics, by age and vaccine formulation selected, Hawaii, USA, 2007–08 influenza season. LAIV, live attenuated influenza vaccine; TIV, trivalent Influenza vaccine; Either, parent or guardian consented to administration of either vaccine formulation to their child. N = 60,694; excludes 66 children for whom vaccine formulation data were not available.

Table 1. Person-hours expended to implement school-located influenza vaccination clinics, Hawaii, USA, 2007–08 influenza season*

	First-dose clinics, n = 345			Second-dose clinics, n = 277			Total, n = 622		
Affiliation or time	Man	Reg	Vac	Man	Reg	Vac	Man	Reg	Vac
PHN	827 (77)	–	1,633 (26)	520 (83)	–	541 (31)	1,347 (80)	–	2,174 (27)
DOH (non-PHN)	155 (15)	4,259 (72)	–	66 (10)	963 (75)	–	221 (13)	5,222 (73)	–
Contract nurses	86 (8)	59 (1)	2,831 (45)	37 (6)	57 (4)	994 (57)	123 (7)	116 (2)	3,825 (48)
Nursing school	–	73 (1)	1,172 (19)	3 (1)	14 (1)	31 (2)	3 (0)	87 (1)	1,203 (15)
Military	–	90 (2)	225 (4)	–	18 (1)	45 (3)		108 (2)	270 (3)
MRC	–	480 (8)	390 (6)	–	115 (9)	118 (7)		595 (8)	508 (6)
Other	–	934 (16)	63 (1)	–	8 (0)	114 (9)		1,048 (15)	70 (1)
Total hours	1,068	5,896	6,313	626	1,281	1,736	1,694	7,177	8,049
Mean hours/clinic	3.1	17.1	18.3	2.3	4.6	6.3	2.7	11.5	12.9

*Man, managers; Reg, registration; Vac, vaccinators; PHN, Public Health Nursing Branch staff, State of Hawaii Department of Health; DOH (non-PHN), other State of Hawaii Department of Health staff, including immunization program staff; nursing school, staff preceptors, and/or nursing school students; military, US Department of Defense healthcare personnel; MRC, Medical Reserve Corps staff from Oahu, Hawaii, Kauai, and Maui Medical Reserve Corps units; other, any other volunteer not affiliated with one of the above organizations. Values are no. (%) unless otherwise indicated.

laid the foundation for an ongoing evaluation of the potential effect of widespread influenza vaccination of school-aged children on the community at large.

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Table 2. Operation costs for statewide school-located influenza vaccination program, Hawaii, USA, 2007–08 influenza season*

Expenditure type	Cost, $	Percentage of total
Vaccine	1,198,403	48
Medical supplies (e.g., adhesive bandage, gloves, alcohol wipes, tissues)	135,000	5
Consent forms, VIS, other print materials	148,278	6
Data management (contracted data entry for consent forms)	42,230	2
Curriculum development and training materials	35,300	1
Public relations; television and radio public service announcements	216,706	9
Courier services to distribute forms and transport vaccine	19,300	1
Student rewards (stickers and hand stamps)	7,000	0
Staffing		
DOH staff hired to implement project	130,199	
Contract nursing services†	266,344	
Estimated cost of in-kind person-hour contributions, e.g., DOH, nursing schools, military, MRC	281,733	
Subtotal for staffing	678,276	*27*
Total	2,480,493	100

*VIS, vaccine information statement; DOH, State of Hawaii Department of Health; MRC, Medical Reserve Corps.
†Contract nursing staff members were procured through an agreement with a private nurse staffing agency; the agency charged the department for a predetermined amount per hour per nurse provided.

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Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007

Eleni Galanis and Laura MacDougall

British Columbia, Canada, has the largest reported population of *Cryptococcus gattii*–infected persons worldwide. To assess the impact of illness, we retrospectively analyzed demographic and clinical features of reported cases, hospitalizations, and deaths during 1999–2007. A total of 218 cases were reported (average annual incidence 5.8 per million persons). Most persons who sought treatment had respiratory illness (76.6%) or lung cryptococcoma (75.4%). Persons without HIV/AIDS hospitalized with cryptococcosis were more likely than those with HIV/AIDS to be older and admitted for pulmonary cryptococcosis. The 19 (8.7%) persons who died were more likely to be older and to have central nervous system disease and infection from the VGIIb strain. Although incidence in British Columbia is high, the predominant strain (VGIIa) does not seem to cause greater illness or death than do other strains. Further studies are needed to explain host and strain characteristics for regional differences in populations affected and disease outcomes.

Cryptoccocus gattii is an environmental fungus that emerged in a temperate climate on Vancouver Island, British Columbia, Canada, in 1999, causing an outbreak affecting humans and animals (*1*,*2*). Previously, *C. gattii* had been reported only from primarily tropical and subtropical regions (*3*,*4*); since then, >200 human cases have been documented in British Columbia.

In British Columbia, *C. gattii* colonizes various species of trees and soil and has been recovered from water and air (*5*). Humans become infected by inhaling yeasts or spores. The primary site of infection is the lung; *C. gattii* can lead to pneumonia or formation of cryptococcomas. The infection can disseminate to most other organs, notably the central nervous system (CNS), where it causes meningoencephalitis or brain cryptococcomas (*6*,*7*). Infection is thought to occur mostly in immunocompetent persons, but new evidence from British Columbia shows that a sizeable proportion of persons with *C. gattii* have underlying immunocompromising conditions (E. Galanis, L. MacDougall, unpub. data). This contrasts with *C. neoformans*, which is distributed worldwide and causes mostly CNS infections in immunocompromised persons, particularly HIV-infected persons (*6*,*7*).

Some researchers have suggested that VGIIa, the predominant strain in British Columbia, is more virulent than other strains found in other countries (*4*,*8*,*9*). Most studies of epidemiologic and clinical aspects of *C. gattii* have shown fewer illnesses and deaths from *C. gattii* infection than from *C. neoformans* (*10*,*11*); however, some have shown they are higher (*12*).

British Columbia has the largest documented population of *C. gattii*–infected persons worldwide. To clarify the epidemiology and impact of illness caused by *C. gattii* infection, we retrospectively analyzed incidence of, hospitalizations for, and deaths caused by *C. gattii* in British Columbia from its emergence in 1999 through 2007.

Methods

Incidence was derived from cases reported to public health authorities. Because complete information about reported cases was not available, hospitalization and death rates were derived from administrative registries.

Case Definitions

BC laboratories report persons infected with *Cryptococcus* spp. to public health authorities (population-based surveillance). We analyzed *C. gattii* infection diagnosed

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during 1999–2007 in BC residents and reported to the BC Centre for Disease Control. A confirmed case was defined as culture-confirmed *C. gattii* infection based on differential media and genotyping (*13*). A probable case was defined as laboratory evidence of infection from antigen detection, histopathology, or microscopy in an HIV-negative person. For their infection to be considered a case, patients must have traveled to or resided in a local *C. gattii*–endemic area during the year before onset. Local *C. gattii*–endemic areas were Vancouver Island since 1999 and the greater Vancouver area and Fraser Valley of the BC mainland since 2004 (*13*). We defined a case acquired on the BC mainland as a confirmed case in a person who did not travel to Vancouver Island or to any international *C. gattii*–endemic area during the year before illness onset.

Case-patients were classified as having a respiratory syndrome if they reported cough or an abnormal chest radiograph or had microbiologic evidence of *Cryptoccocus* in a respiratory specimen. Case-patients were classified as having a CNS syndrome if they had abnormal brain imaging or microbiologic evidence of *Cryptococcus* in a brain or cerebrospinal fluid specimen. Case-patients with other presentations had microbiologic evidence of *Cryptococcus* in another organ/tissue. Case-patients who were HIV positive or had a history of invasive cancer or organ transplant or were on corticosteroids in the year before onset were considered immunocompromised.

Incidence, Epidemiology, and Clinical Characteristics

Incidence, epidemiology, and clinical characteristics were analyzed for confirmed, probable, and all cases and compared between confirmed and probable cases. We derived incidence using annual BC population estimates. We calculated frequencies using the case-patients from whom the information was available, which varied for each question or data point.

Hospitalizations

Hospital discharge data for International Classification of Diseases, Ninth Revision (ICD-9), code 117.5 and Tenth Revision (ICD-10) code B45.X (B45.0, B45.1, B45.2, B45.3, B45.7, B45.8, B45.9) were obtained from the BC Hospital Separations/Discharge Abstract Database, which captures all hospital visits in the province, for 1999–2006 (*14*). (British Columbia switched from ICD-9 to ICD-10 coding in 2001.) Records with the same unique identifier and age (adjusted for date of hospitalization) were considered to represent the same person because unique identifiers for certain types of patients were recycled. Annual hospitalization rates were derived by adding the earliest hospitalization for each individual and dividing by the annual BC population. Additional hospitalizations for the same person were considered repeat hospitalizations.

Because ICD-9 and ICD-10 have no *C. gattii*-specific code, we analyzed persons hospitalized with cryptococcosis without HIV/AIDS. We chose this population as a proxy for *C. gattii* infection because its hospitalization rate increased sharply in 1999, signaling onset of the outbreak (*1*). In addition, few (6.2%) confirmed *C. gattii*–infected persons have HIV infection in British Columbia (E. Galanis, L. MacDougall, unpub. data). HIV/AIDS was defined as ICD-9 codes V08 and 042.X (42.0, 42.1, 42.2, 42.8, 42.9) and ICD-10 codes B20, B21, B22, B23, and B24. Cases of cryptococcosis were separated into those with no ICD code for HIV/AIDS reported on any hospitalization record within the study period and those with an ICD code for HIV/AIDS reported at least once. Among cases with an ICD-10 code for cryptococcosis, subcodes were analyzed. (Subcodes for cryptococcosis are not available in ICD-9.)

Deaths

Non-nominal data for deaths from cryptoccocal infection were obtained from BC Vital Statistics, which includes all deaths among BC residents (*15*). All deaths occurring during 1999–2007 for which the underlying or a contributing cause of death was noted as ICD-9 code 117.5 or ICD-10 code B45.X were extracted. We identified deaths in persons with *C. gattii* infection by matching birth date with cases reported to the BC Centre for Disease Control. We derived annual death rates by dividing the number of deaths by the annual BC population.

Population Data, Analysis and Software Used, and Patient Consent

Population data were obtained from British Columbia Statistics (*16*). Using the χ^2 test and Fisher exact test, we compared independent proportions; the t test, to compare means; and the Mann-Whitney test, to compare non-normally distributed results. We used the Bonferroni correction to compare strain distribution by age group. Data were analyzed by using SPSS v16.0 (SPSS Inc., Chicago, IL, USA) and StatXact-6 v6.2.0. (StataCorp LP, College Station, TX, USA) Because the data were obtained from surveillance and administrative sources and presented in aggregate format, patient consent was not obtained.

Results

Incidence, Epidemiology, and Clinical Characteristics

A total of 218 cases (124 confirmed and 94 probable) of *C. gattii* infection were reported during 1999–2007 (Table 1). An average of 24.2 cases was reported every year (5.8/million/year); cases increased steadily from 6 in 1999 to 38 in 2006 (Figure 1). Onset did not vary by season or by month. Nearly three quarters (73.9%) of all case-patients lived on Vancouver Island (average annual incidence

Table 1. Characteristics of persons with cases of confirmed or probable *Cryptococcus gattii* infection, British Columbia, Canada, 1999–2007*

Characteristic	Total	Confirmed	Probable	p value
No. cases	218	124	94	NA
Average incidence (million/year)	5.8	3.3	2.5	NA
No. cases in persons living on Vancouver Island	161 (73.9)	83 (66.9)	78 (83.0)	0.177
Demographic data				
Male sex	121 (55.5)	73 (58.9)	48 (51.1)	0.440
Age, y				
Mean	58.7	58.7	58.7	0.988
Range	2–92	2–92	12–87	NA
Children <18 y	4 (1.8)	3 (2.4)	1 (1.1)	0.463
Clinical assessment				
Respiratory syndrome	167 (76.6)	85 (68.5)	82 (87.2)	0.031
CNS syndrome	17 (7.8)	16 (12.9)	1 (1.1)	0.001
Respiratory and CNS syndrome	22 (10.1)	20 (16.1)	2 (2.1)	<0.001
Other/unknown	12 (5.5)	3 (2.4)	9 (9.6)	NA
Asymptomatic	16 (7.3)	6 (4.8)	10 (11.0)	0.120
Hospitalized	98 (60.9)	58 (65.2)	40 (55.6)	0.434
Immunocompromised	70 (38.0)	41 (38.7)	29 (37.2)	0.870

*Frequencies were calculated by using persons from whom information was available, which varied for each question or data point. All values given as no. (%) persons except as indicated. NA, not available; CNS, central nervous system.

rate 25.1/million). The number of cases reported per year reached a plateau on Vancouver Island in 2002 but has increased on the mainland since 2005. Seven confirmed cases were acquired on the BC mainland (1 in 2004, 2 in 2005, 3 in 2006, and 1 in 2007).

Just over half (55.5%) of all case-patients were male. The mean age was 58.7 years. The incidence rate increased with age, with the highest age-specific rate in persons 70–79 years of age.

A total of 167 (76.6%) case-patients sought treatment for a respiratory syndrome; 17 (7.8%), for a CNS syndrome; 22 (10.1%), for both respiratory and CNS syndromes; and 1 each, for a combination of skin and respiratory, skin and CNS, and sepsis and respiratory syndrome (Table 1). The most common symptoms reported in case-patients with a respiratory syndrome were cough, dyspnea, and chest pain. Case-patients with a CNS syndrome most commonly reported headache, night sweats, weight loss, anorexia, and neck stiffness. Eighty-nine (75.4%) of the118 case-patients who had abnormal chest radiographs had single or multiple lung nodules. Sixteen (7.3%) cases were asymptomatic. All had a respiratory syndrome, and all who reported chest radiograph results had evidence of single or multiple lung nodules. Age, sex, and genotype did not differ significantly from those of symptomatic cases. Ninety-eight (60.9%) case-patients were hospitalized. Seventy (38.0%) case-patients were considered immunocompromised; 81.4% had a respiratory syndrome and 5.7% had CNS signs. Being immunocompromised was not associated with clinical presentation (p = 0.385).

Patients with confirmed and probable cases did not differ significantly by age and sex or proportions residing on Vancouver Island, asymptomatic, hospitalized, or immunocompromised (Table 1). Persons with confirmed cases were more likely than persons with probable cases to have a CNS syndrome only (p = 0.001) or a CNS and respiratory syndrome (p<0.001) and less likely to have a respiratory syndrome only (p = 0.031).

Three confirmed cases occurred in children 2, 5, and 16 years of age. Each had a respiratory syndrome; 1 was asymptomatic. All were HIV negative. Two were on inhaled corticosteroids. One had a chronic respiratory disease, 1 had a genetic disorder; the third was otherwise well.

The VGIIa strain was responsible for 107 (86.3%) of confirmed cases. Eight case-patients were infected with VGI and 9 with VGIIb. Clinical presentation did not differ significantly by genotype. However, strains differed by patient age; case-patients ≥50 years of age were more likely to be infected with VGIIa (p = 0.002) or VGIIb (p = 0.006) than with VGI (Figure 2).

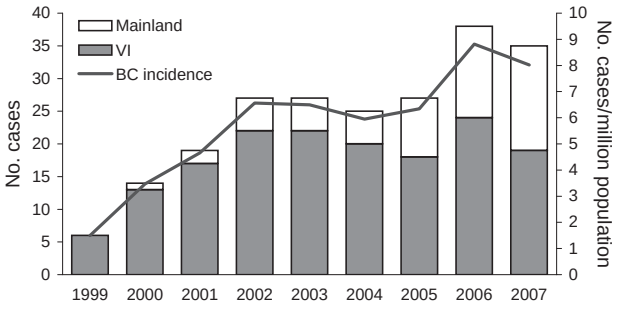

Figure 1. Number of cases of *Cryptococcus gattii* infection and incidence rate per million population, by case-patient place of residence, British Columbia (BC), Canada, 1999–2007. Mainland, mainland BC; VI, Vancouver Island.

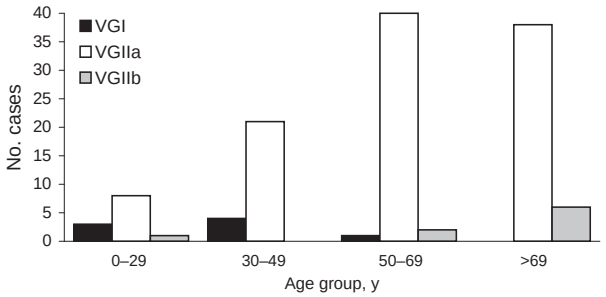

Figure 2. Distribution of *Cryptococcus gattii* strains among 124 persons with *C. gattii* infection, by age category, British Columbia, Canada, 1999–2007.

Hospitalizations

During 1999–2006, a total of 322 persons were hospitalized with cryptococcosis, for a total of 533 hospitalizations. A total of 191 (59.3%) persons did not have HIV/AIDS. An average of 21.2 persons without HIV/AIDS were hospitalized for cryptococcosis each year; this number increased steadily from 10 in 1999 to 38 in 2006 (Figure 3). At 5.8 hospitalizations per million BC residents, the average annual hospitalization rate was higher for cryptococcosis without HIV/AIDS than with HIV/AIDS ($p = 0.004$) (Table 2). Persons with HIV/AIDS had more admissions per person and were hospitalized for longer periods (both $p<0.001$). Hospitalized persons without HIV/AIDS were less likely to be male ($p = 0.007$) and more likely to be older ($p<0.001$) than those with HIV/AIDS.

Among cryptococcosis hospitalizations for which an ICD-10 code was available, most (56.5%) hospitalizations for persons without HIV/AIDS were for pulmonary cryptococcosis; most (77.7%) hospitalizations for persons with HIV/AIDS were for cerebral cryptococcosis (Table 3). Persons without HIV/AIDS were more likely to be hospitalized with pulmonary ($p<0.001$) or cutaneous ($p = 0.044$) disease but less likely to be hospitalized with cerebral ($p<0.001$) or disseminated ($p<0.001$) disease than were persons with HIV/AIDS.

Deaths

During 1999–2007, 19 case-patients (case-fatality ratio [CFR] 8.7%) died from or with *C. gattii* infection, of which 15 cases were confirmed (CFR = 12.1%). The *C. gattii*–specific average annual death rate, based on all deaths, was 0.5/million. During the same period, 42 case-patients died with cryptococcosis and HIV/AIDS infection, for an average annual death rate of 1.1/million in British Columbia.

Thirteen (68.4%) case-patients who died were male, but this percentage did not significantly differ from that of case-patients who survived ($p = 0.474$). Mean age at death was 67.6 years (range 26–91 years). Persons who died were older at diagnosis than survivors ($p = 0.019$) and more likely to have CNS syndrome with or without respiratory syndrome ($p = 0.014$). Ten (66.7%) persons with confirmed cases who died had an infection caused by VGIIa; 4 (26.7%), by VGIIb; and 1 (6.7%), by VGI. Case-patients who died were more likely to have been infected by VGIIb than by the other 2 strains combined ($p = 0.002$).

On the basis of public health interviews and death certificates, 14 (73.7%) of all case-patients who died had underlying medical conditions that might have increased their risk for death, including cancer, chronic obstructive pulmonary disease, asthma, liver disease, diabetes, HIV infection, lung transplant, congestive heart failure, and congenital heart malformation. Nine (47.4%) were immunocompromised; case-patients who died were not more likely than survivors to be immunocompromised ($p = 0.267$).

Discussion

We have presented a population-based assessment of the epidemiology and impact of illness of *C. gattii* infection in a newly endemic part of the world. Although a number of studies have assessed epidemiologic and clinical features of cryptococcosis, few have done so for *C. gattii* cases separately, particularly in recent years and outside Australia (*10–12*,*17*). Because few regions other than British Columbia and France have ongoing population-based surveillance for cryptococcosis, most previous studies were based on retrospective hospital chart reviews or surveys (*18*).

The average annual incidence of *C. gattii* infection on Vancouver Island (25.1/million) is one of the highest in the world. Australia reported an annual cryptococcal infection incidence of 140/million in Aboriginals in Arnhemland, Northern Territory, in 1976–1992 (*19*); a total of 77.8% of these cases were caused by *C. gattii*. Papua New Guinea reported an annual *C. gattii* incidence of 42.8/million in the Central Province in 1993–1995, but this included cases in

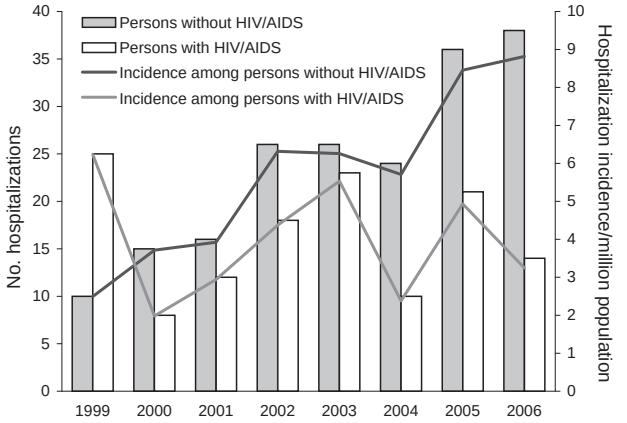

Figure 3. Comparison of hospitalizations for cryptococcosis among persons with and without HIV/AIDS, British Columbia, Canada, 1999–2006.

Table 2. Characteristics of hospitalizations and demographic data for 322 persons hospitalized for cryptococcosis, British Columbia, Canada, 1999–2006

Characteristic	Cryptococcosis without HIV/AIDS	Cryptococcosis with HIV/AIDS	p value
No. hospitalizations	266	267	
No. case-patients	191	131	
Incidence*	5.8	3.9	0.004
No. admissions per case-patient, median (range)	1 (1–7)	3 (1–11)	<0.001
Length of stay, d, median (range)	8 (0–142)	14 (1–288)	<0.001
Demographic data			
Male sex, %	56.5	82.4	0.007
Age, y			
Mean	57.9	40.3	<0.001
Range	1–91	6–75	

*Average annual hospitalization rate per million British Columbia population.

residents of other provinces (*20*). The reason for the high incidence in British Columbia and these other regions is unclear but may be due to ecologic, host, or strain-related characteristics.

The incidence in British Columbia increased in the early years of the emergence (1999–2001), either because of increasing awareness and reporting or a true increase in incidence from increased fungal concentration over time or gradual cumulative infection of exposed susceptible persons. The incidence on Vancouver Island stabilized in 2002, but the overall BC incidence increased in 2006–2007 because of an increased number of case-patients residing on the BC mainland, suggesting that the true number of persons exposed there may be higher than we estimated using our specific case definition for acquisition on the mainland.

Although the definition for a confirmed case is more specific, all variables analyzed are similar for confirmed and probable cases except clinical presentation. Cases that include a CNS syndrome may be more likely to be confirmed through a cerebrospinal fluid culture than are cases that include respiratory syndrome, which require a more invasive pulmonary sample for culture confirmation.

C. gattii incidence rates and trends in British Columbia are similar to the cryptococcosis hospitalization rate for persons without HIV/AIDS. However, only 60.9% of *C. gattii*–infected case-patients reported being hospitalized for their illness. This discrepancy most likely results from hospitalization of persons with other immunocompromising conditions whose cryptococcal infection was caused by *C. neoformans* rather than *C. gattii*. Before *C. gattii* emerged in 1999, an average of 11 persons were hospitalized with cryptococcosis without HIV/AIDS in British Columbia each year (*1*). This average is similar to the annual incidence of cryptococcosis in persons without HIV/AIDS in non–*C. gattii*–endemic regions (*7*,*21*–*23*). Therefore, the true *C. gattii* hospitalization rate is probably lower than that reported here. Lack of a *C. gattii*–specific ICD code and exclusion of persons hospitalized before 1999 limited our assessment.

Most (90.8%) *C. gattii*–infected persons sought treatment for respiratory syndrome with or without neurologic findings. Respiratory presentations and pulmonary cryptococcomas are commonly associated with *C. gattii* or cryptococcal infections in immunocompetent patients (*10*,*24*). Only 18.3% of BC cases had evidence of CNS involvement initially, which contrasts with an Australian study of hospitalized patients in which 85% of 20 *C. gattii*–infected patients had meningitis and a nationwide survey in Colombia in which 93.3% of 30 had neurologic findings (*10*,*17*). Also in contrast to other authors, we found that case-patients without HIV/AIDS were admitted less often and hospitalized for less time than those with HIV/AIDS (*12*). These differences may be due to the timing of data collection in

Table 3. ICD-10 codes for cryptococcosis hospitalizations, British Columbia, Canada, 1999–2006 (N = 322)*

Condition (ICD-10 code)	Cryptococcosis without HIV/AIDS, no. (%)†	Cryptococcosis with HIV/AIDS, no. (%)†	p value
Pulmonary cryptococcosis (B45.0)	130 (56.5)	13 (6.7)	<0.001
Cerebral cryptococcosis (B45.1)	63 (27.4)	150 (77.7)	<0.001
Cutaneous cryptococcosis (B45.2)	7 (3.0)	1 (0.5)	0.044
Osseous cryptococcosis (B45.3)	1 (0.4)	0	ND
Disseminated cryptococcosis (B45.7)	5 (2.2)	22 (11.4)	<0.001
Other forms of cryptococcosis (B45.8)	11 (4.8)	6 (3.1)	0.289
Cryptococcus, unspecified (B45.9)	13 (5.7)	12 (6.2)	0.834
Missing ICD-10 code	36	74	
Total	266	267	

*ICD-10, International Classification of Diseases, tenth revision; ND, not determined.
†Total hospitalizations minus hospitalizations for which ICD code is missing.

the progression of disease (which may have occurred earlier in British Columbia); the fact that the case-patients in the Australian studies were all hospitalized (and probably more seriously ill); and differences in study methods, medical practices, and strain characteristics. Our analysis was limited by the self-reported nature of the data, which lacked specificity and detail.

Published estimates of CFRs vary widely; from 0% of 20 cases to 15% of 26 cases with CNS disease in Australia (*10*,*12*). The CFR in British Columbia (8.7%) is probably a more stable estimate given the large number of case-patients for whom information was available and, possibly, the different strain distribution. BC case-patients who died were more likely to be older, have CNS disease, and be infected with VGIIb. The small numbers preclude assessment of whether death was independently associated with age or genotype. Other studies also have found that CNS disease and age increase the risk for death (*12*,*25*).

Of *C. gattii*-infected persons, 7.3% (9.6% of cases with a respiratory syndrome) were asymptomatic. The few studies describing this finding report that approximately one third of pulmonary cryptococcal cases are asymptomatic (*24*,*26*,*27*). The much lower proportion of asymptomatic cases in British Columbia may result from species or strain differences or diagnostic and reporting practices.

In British Columbia, 3 (2.4%) persons with confirmed cases were <18 years of age. In most *C. gattii*–endemic areas except northern Brazil, cryptococcosis in children is rarely reported (*12*,*17*,*28*–*30*). Two of the BC pediatric case-patients had underlying conditions affecting their lungs and were on inhaled corticosteroids. Diminished respiratory function or some level of immunocompromise may be necessary for children to become infected with *C. gattii*. The natural history of disease remains unclear; symptomatic disease may be associated with recent exposure, prolonged incubation, or reactivation of latent disease (*31*,*32*). In British Columbia, the incidence is highest in the 70–79-year age group, which may be linked to underlying medical conditions or to decreasing age-related cellular immunity, both of which could lead to acute infection or reactivation of latent disease.

Mouse models show that VGIIa may be more virulent than VGI and *C. neoformans* (*9*). Although *C. gattii* incidence is comparatively high in British Columbia, we could not find evidence that VGIIa causes more severe illness and a higher death rate than do other strains or *C. neoformans*. In British Columbia, persons hospitalized with cryptococcosis and HIV/AIDS (a proxy for *C. neoformans* infection) were more likely than those without HIV/AIDS to be hospitalized with severe disease. The death rate for case-patients with HIV/AIDS was twice as high as that for persons with *C. gattii* infection. In British Columbia, *C. gattii*–infected persons were less likely to present with CNS disease and no more likely to die from their infection than those in Australia, most of which is caused by VGI. In addition, we found that VGIIb infections may be more likely to be associated with a fatal outcome than VGIIa or VGI infections, either directly or because they affect older persons. This finding might be related to a strain-specific ability to reactivate. This association merits further study. To further assess the pathogenicity of *Cryptococcus* strains, common genotyping methods need to be used routinely to compare strains and outcomes internationally.

Evidence is increasing that *C. gattii* affects different populations and has a different clinical presentation and outcome than *C. neoformans* infection. Whether this is due to strain or host characteristics remains unclear. Speciation of *Cryptococcus* and laboratory-based surveillance should be considered for all areas where *C. gattii* is known to be, or is possibly, endemic. Where the disease is not reportable, a *C. gattii*–specific ICD code would allow surveillance of hospitalized case-patients. Raising awareness among physicians is necessary to ensure appropriate specimens are collected for culture and diagnoses are accurately reported.

Understanding of the progression of *C. gattii* infection and disease is lacking. *C. gattii*–specific serologic tools and long-term studies are needed to better understand natural progression and factors that impact outcome to better manage the risk associated with *C. gattii* and patients affected by it.

We have provided evidence of substantial *C. gattii*–related illness and of continued yet limited acquisition on the BC mainland since 2004. With its recent identification in the US Pacific Northwest, standardized laboratory-based surveillance and sharing of epidemiologic data are necessary to increase understanding of how and where this elusive pathogen spreads (*13*,*33*–*35*).

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Tropheryma whipplei in Patients with Pneumonia

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Tropheryma whipplei is the etiologic pathogenic agent of Whipple disease (WD), characterized by various clinical signs, such as diarrhea, weight loss, lymphadenopathy, and polyarthritis. PCR-based methods for diagnosis of WD have been developed. *T. whipplei* has been identified in saliva and stool samples from patients with WD and from healthy persons. *T. whipplei* DNA has also been found in bronchoalveolar lavage (BAL) samples of a child with pneumonia. We detected DNA of *T. whipplei* in 6 (3%) of 210 BAL samples collected in intensive care units by using 16S rDNA and specific quantitative PCR. We identified 4 novel genotypes of *T. whipplei*. In 1 case, *T. whipplei* was the only bacterium; in 4 others, it was associated with buccal flora. We suggest that *T. whipplei* should be investigated as an etiologic agent of pneumonia.

Tropheryma whipplei is a bacterium widely known to be associated with Whipple disease (WD), which is characterized by various clinical signs such as diarrhea, weight loss, lymphadenopathy, and polyarthritis (*1*). Furthermore, clinical features such as blood culture–negative endocarditis and neurologic lesions have been reported (*2*). Until 1991, WD diagnosis was based essentially on histopathologic observations, characterized by positive periodic acid-Schiff stained inclusions within intestinal macrophages (*3*) and electron microscopy, which has shown the microbiologic etiology of the illness (*4*).

The first molecular identification was made by Wilson et al. in 1991 (the bacterium was uncultured at that time) by using broad-range primers targeting the 16S subunit of rDNA extracted from infected duodenal tissue of a patient with WD (*5*). The bacteria were thus classified within the family *Actinomycetes* (classification was based on 16S rDNA sequence analysis), and the name *Tropheryma whippelii* was proposed (*5*,*6*); the bacterium was later modified to *Tropheryma whipplei* by La Scola et al. (*7*). Investigators then developed several molecular PCR-based methods for the diagnosis of WD. Most of these methods use probes or primer sets targeting parts of the 16S or 23S rDNA genes (*8*).

Successful cultivation of the bacterium by Raoult et al. (*9*) has enabled the adoption of novel diagnostic tools, such as immunohistochemistry and serologic tests (*10*–*12*). Three years after successful cultivation, the genomes of 2 *T. whipplei* strains (T–W08/17, GenBank accession no. NC004551, and Twist, GenBank accession no. NC004572) have been fully sequenced (*13*,*14*). Completion of the sequencing of these 2 genomes has enabled the design of highly specific and sensitive primer pairs that target repeated DNA sequences unique to the *T. whipplei* genome (*15*).

These major advances have enabled the identification of *T. whipplei* DNA in various specimens such as saliva and stools from patients with WD, as well as from asymptomatic carriers, which suggests that the localization of the bacterium is not restricted to the digestive tract and that other organs might be affected (*2*,*16*–*18*). More recently, detection of DNA from *T. whipplei* in bronchoalveolar lavage (BAL) samples from a patient with pneumonia suggests that the bacterium might be involved in respiratory diseases (*19*). We show that *T. whipplei* could be a potential infectious agent for patients admitted to intensive care units (ICUs) and that it can be found in the BAL samples of patients in ICUs.

Materials and Methods

All case-patients (hereafter patients) were admitted to 1 of 3 ICUs in Marseille, France (1 medical ICU and 2

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medicosurgical ICUs) during February 2007 through January 2008. Ages ranged from 18 to 94 years. A total of 210 bronchoalveolar lavage (BAL) fluid samples and 197 blood samples, representing 197 episodes of suspected or confirmed pneumonia, were collected from 134 patients admitted to the 3 ICUs to perform an exhaustive etiologic diagnosis of pneumonia. Bronchoalveolar lavage and blood sampling were collected as previously described (*20*) in the 3 ICUs. The samples were then transported at room temperature to the microbiology laboratory of Timone Hospital, Marseille, France, and preserved at –20°C until handling. Ventilator-associated pneumonia, community-acquired pneumonia, and aspiration pneumonia were defined as previously described (*21–23*).

DNA was extracted in a MagnaPure LC workstation (Roche Diagnostics, Meylan, France) by using the MagNa Pure LC DNA Isolation Kit II (Roche Diagnostics). Pellets from BAL fluid samples were mixed with 200 μL of lysis buffer and 50 μL of proteinase K, incubated overnight at 56°C, disrupted for 1 min with glass beads in a MagNa Lyser (Roche Diagnostics), and then processed on the Magnapure LC workstation by the manufacturer's recommendations. When a BAL sample was cell-poor, to concentrate the bacterial cells, we centrifuged 500 μL of BAL sample for 15 min at maximum speed, discarded 400 μL of the supernatant, then resuspended the pellet in the remaining 100 μL; the extraction procedure was continued as described above.

Standard PCR was performed by using the eubacterial broad-range 16S rDNA primer set 536F: 5′-CAGCAGCCGCGGTAATAC-3′ and rp2: 5′-ACGGC TACCTTGTTACGACTT-3′ (Eurogentec, Seraing, Belgium). PCR was performed in an ABI Themocycler (Applied Biosystems, Courtaboeuf, France).The amplification was done in a 50-μL final volume containing 5 μL extracted DNA, 1X PCR buffer (5 μL), 2 μL of 25 mM $MgCl_2$, 200 μM of each dNTP, 0.2 mM of each 536F and rp2 primer, and 1 unit of HotStar Taq DNA Polymerase (QIAGEN, Courtaboeuf, France). Amplification started with an initial incubation at 95°C for 15 min to denature DNA and activate polymerase enzymes, followed by 35 cycles of heating at 95°C for 1 min, annealing at 62°C for 30 s, and extension at 72°C for 90 s. Amplification ended with an extension step at 72°C for 10 min. The PCR products were purified by using the Nucleo-Fast 96 PCR Kit (MACHEREY-NAGEL, Hoerdt, France) as defined by the supplier, and 4 μL of purified PCR product was sequenced in 20 μL final volume containing sequencing buffer, 3.2 pmole of forward (536F) or reverse (rp2) primer, 3 μL of Big Dye Terminator V1.1 mix (Applied Biosystems), and 8 μL of deionized water. Sequencing reactions were purified by using Sephadex Gel-Filtration (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), and the purified products were sequenced on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). Sequences obtained were analyzed with Autoassembler software and compared with those available in the GenBank database by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST).

When the BAL fluid was polymicrobial, purified PCR products were cloned into PCR II TA cloning vector (Invitrogen, Cergy Pontoise, France) by using 3 μL of purified PCR products from the previous step as recommended by the manufacturer, and 56–64 white colonies were screened for each specimen. The cloned inserts were amplified with M13 primers set (M13F: 5′-GTAAAACGACGGCCAG-3′, M13R: 5′-CAGGAAACAGCTATGAC-3′) and sequenced as described above.

Quantitative real-time PCR was performed as described by the manufacturer by using a LightCycler instrument (Roche Diagnostics) with the QuantiTect Probe PCR Kit. First, specimens were tested by using the Twhi3F: 5′-TTGTGTATTTGGTATTAGATGAAACAG-3′ and Twhi3R: 5′-CCCTACAATATGAAACAGCCTTTG-3′ primer pair and the specific TaqMan probe Twhi3: 6-FAM-GGGATAGAGCAGGAGGTGTCTGTCTGG-TAMRA. When the specimen was positive in this assay, the result was confirmed by a second quantitative PCR by using the Twhi2F: 5′-TGAGGATGTA TCTGTGTATGGGACA-3′ and Twhi2R: 5′-TCCTGT TACAAGCAGTACAAAACAAA-3′ primer set and the Twhi2 probe: 6-FAM-GAGAGATGGGGTGCAGGACA GGG-TAMRA.

Genotyping of *T. whipplei* detected in the BAL fluid samples was conducted as described previously (*24*). Each of the 4 highly variable genomic sequences (HVGSs) obtained from each specimen was compared with those available in the GenBank database and our internal laboratory database to determine their corresponding genotype. The combination of the 4 HVGSs was then analyzed to define the genotype of the bacteria.

Serologic assays were performed by Western blotting. The *T. whipplei* Twist strain was cultivated in axenic medium as previously reported (*25*). Native and deglycosylated samples obtained from the total bacterial extract were prepared for sodium dodecyl sulfate–polycrylamide gel electrophoresis. The assay was performed to test all immunoglobulins (Ig), total (IgT), including IgG, IgM, and IgA heavy and light chains, as well as to test IgG, IgM, and IgA separately, as previously described (*26*). Detection was performed by using chemiluminescence (ECL Western Blotting Analysis System) and an automated film processor (Hyperprocessor, GE Healthcare, Buckinghamshire, UK). To quantify Western blot bands, we scanned films with an Image Scanner III (GE Healthcare). We performed image analysis by using GelEval 1.21b FrogDance software and ImageJ software (http://rsb.info.nih.gov/ij).

Results

Bacterial DNA of *T. whipplei* was detected in 6 of 210 BAL fluid samples by standard or quantitative PCR (Table 1). The patients' ages ranged from 39 to 73 years (mean ± SD 56.83 ± 14.01 years), and all were men (Table 2). One of those 6 specimens (no. 5) was positive for the bacterium in both standard and quantitative PCR assays. For this patient, PCR with broad-range primers showed that *T. whipplei* was the only bacterium identified. Moreover, both quantitative PCR assays showed a high level of the bacterium in this specimen (cycle threshold = 20 with Whi3 probe and cycle threshold = 21 with Whi2 probe; 5.10^5 copy [Table 1]). This patient was immunocompromised and had community-acquired pneumonia and septic shock (Table 2). He was admitted to the medical ICU for septic shock and acute respiratory distress syndrome complicating community-acquired pneumonia (Figure). He received chemotherapy for difficult-to-treat mediastinal lymphoma. A lobectomy on his upper right lung had been done 1 year before admission for the lymphoma. Lung infiltrates were present when he was admitted to the hospital. The patient was febrile (39.2°C) and hypoxemic (partial pressure of oxygen in arterial blood [PaO_2] 120 mm Hg for a fraction of inspired oxygen [FiO_2] at 1), and pancytopenia was evident after initial examination. He received blood products during his ICU stay (9 packed erythrocytes, 4 fresh frozen plasma, 3 platelet transfusions). He was empirically treated by using ticarcillin/clavulanic acid and erythromycin. Hemodynamic status improved rapidly, and administration of vasopressors was stopped by day 2. The patient was finally extubated at day 7 and discharged from the ICU on day 10. He fully recovered after completing a treatment regimen of imipenem, amikacin, and vancomycin.

Specimens nos. 10, 82, 86, 183, and 209 from 5 other patients were positive for *T. whipplei* in quantitative real-time PCR by using the Twhi3 probe and primer pairs and successfully confirmed by using the Twhi2 probe and primer pairs (Table 1). The bacterial load in each specimen is shown in Table 1. Standard cultures were negative; 10^4 CFUs were used as the cutoff. The 16S rDNA assay yielded 50 bacteria representing 46 species (Table 1). Of these 46 bacterial species, 15 had ≤97% 16S rRNA sequence identity with the sequences available in the GenBank database, which suggests a potentially new bacterial species (Table 1). However, despite the high number of the clones screened for these patients, this approach was unable to detect DNA of *T. whipplei*. This result could be explained by the polymicrobial aspect of these specimens caused by the presence of oral bacterial flora, which was proven by the high number of bacteria identified from each specimen (Table 1). Patients 10, 82, 86, and 183 had a typical buccal flora. Two of these 4 patients had aspiration pneumonia; the remaining 2 had ventilator-associated pneumonia. The patient from whom specimen no. 209 was collected had flora of an apparent environment and water origin, but aspiration pneumonia likely developed.

Genotyping of *T. whipplei* was successfully performed for 4 specimens (specimen nos. 5, 10, 82, and 86). Three new *T. whipplei* genotypes were identified in specimen nos. 5, 82, and 86. *T. whipplei* genotype 3 was isolated from specimen 10; this genotype is currently identified in patients with endocarditis caused by *T. whipplei* and in patients with digestive WD (*24*). Another new genotype of *T. whipplei* was identified in specimen no. 183. In this sample, because of the low level of the bacterium, only 2 HVGSs (HVGS1 and HVGS3) were successfully amplified. However, after analysis of the HVGS1 sequence, we concluded that it was a new genotype different from those previously identified. Serologic assays showed no immunoreactivity in the serum of patients whose BAL samples were positive for *T. whipplei* in molecular assays.

Discussion

We report the use of broad-range primer-based PCR amplifying the 16S rDNA gene followed by cloning and sequencing to identify bacteria associated with pneumonia occurring in mechanically ventilated ICU patients. These results agree with previously published studies (*19*,*27*,*28*) of cystic fibrosis patients, which showed that unusual microbiologic agents can be responsible for pneumonia. The present study shows that these unusual agents of pneumonia can also be identified in mechanically ventilated ICU patients.

Our results show that *T. whipplei* is an etiologic pathogen in pneumonia. We tested samples from all patients positive for *T. whipplei* twice by RTq PCR using different primer pairs and probes. In the broad-range PCR-based assay, samples from the first patient were positive for *T. whipplei* by direct amplification and sequencing of the 16S rDNA gene. Together with the published work of Harris et al., this positive result prompted us to test systematically for *T. whipplei* in all BAL samples collected (*19*). Indeed, as we were conducting our study, Harris et al. reported the detection of *T. whipplei* in the sputum of a child with pneumonia (*19*). Moreover, we were able to genotype *T. whipplei* in 5 specimens (i.e., 4 further amplifications for nos. 5, 10, 82, and 86 and 2 other amplifications for no. 183). Serologic assays showed no immunoreactivity in the serum of the patients of interest. In this age group (61 ± 3.6 years), the percentage of positivity by Western blot is 45% (F. Fenollar, D. Raoult, unpub. data). The absence of immunoreactivity in serum of patients whose BAL fluid was positive for *T. whipplei* in molecular assays shows that *T. whipplei* was probably not present in those patients before the current pneumonia developed. Moreover, antibody response against *T. whipplei* is paradoxal. We recently

Table 1. PCR test results of bronchoalveolar lavage specimens that were positive for *Tropheryma whipplei* DNA, collected from 6 intensive care unit patients in Marseille, France, February 2007–January 2008*

Specimen no.	16S rDNA for *T. whipplei*	qPCR probe					Other bacteria identified (16S rDNA primer set)	
		Twhi3	Ct	Twhi2	Ct	Load (copy)	Bacterium	Identity, %
5	+	+	20	+	21	5.10^5	–	99
10	–	+	30	+	31	$4,8.10^2$	*Streptococcus pneumoniae*	99
							Actinobacillus pleuropneumoniae	99
							Peptostreptococcus sp.	99
82	–	+	29	+	28	$5,3.10^3$	*Streptococcus* genomosp. C4	99
							Uncultured	99
							Streptococcus sp. clone 2.17	89
							Streptococcus sanguinis	99
							Gemella sanguinis	99
							Leptotrichia sp.	95
							Haemophilus quentini	99
							Uncultured *Haemophilus* sp.	99
							Peptostreptococcus sp.	99
							Granulicatella para-adiacens	95
							Prevotella sp.	99
							Prevotella melaninogenica	99
							Cloacibacterium normanense	93
							Ralstonia solanacearum	99
							Haemophilus haemolyticus	99
							Uncultured *Arcobacter* sp. clone DS126	99
							Prevotella salivae	99
							Streptococcus parasanguis	99
86	–	+	31	+	29	$7,6.10^2$	Uncultured *Porphyromonas* sp. clone 302E06	99
							Uncultured *Capnocytophaga* sp.	95
							Gemella sanguinis	99
							Streptococcus constellatus	99
							Prevotella melaninogenica	91
							Uncultured *Porphyromonas* sp.	93
							Haemophilus parainfluenzae	99
							Uncultured *Tannerella* sp.	98
							Prevotella melaninogenica	99
							Granulicatella para-adiacens	99
183	–	+	36	+	37	50	*Staphylococcus epidermidis*	99
							Porphyromonas gingivalis	99
							Pseudomonas sp.	92
							Eubacterium sp.	94
							Streptococcus anginosus	99
							Uncultured *Neisseria* sp. clone 502G08	99
							Treponema sp.	97
							Clostridium sp.	97
							Acidovorax sp./*Diaphorobacter* sp.	99
							Eubacterium brachy	99
							Gemella haemolysans	99
							Mycoplasma orale	95
							Comamonas denitrificans	95
							Prevotella tannerae	98
							Peptostreptococcus micros	99
209	–	+	35	+	35	70	*Abiotrophia defectiva*	99
							Acidobacteria sp.	94
							Afipia genosp. 12	97
							Sphingomonas sp.	98
							Pseudomonas stutzeri	100

*qPCR, quantitative PCR; Ct, cycle threshold.

Table 2. Demographic and clinical data on 6 intensive care unit patients with pneumonia from whom bronchoalveolar lavage samples positive for *Tropheryma whipplei* DNA were collected, Marseille, France, February 2007–January 2008*

Specimen no.	Patient age, y/sex	Immunocompromised	Diagnosis	Type of pneumonia	Duration of MV, d	Duration of ICU stay, d	*T. whipplei* genotype	Outcome
5	39/M	Yes (chemotherapy)	Septic shock	CAP	7	10	New	Survived
10	46/M	Yes (splenectomy)	Coma	Aspiration	3	5	3	Survived
82	65/M	No	Coma	Aspiration	16	27	New	Survived
86	74/M	No	Pulmonary embolism	VAP	42	42	New	Died
183	43/M	No	Pancreatitis	VAP	14	16	New	Survived
209	74/M	Yes (corticosteroids)	Spinal cord injury	Aspiration	81	81	ND	Died

*MV, mechanical ventilation; ICU, intensive care unit; CAP, community-acquired pneumonia; VAP, ventilator-associated pneumonia; ND, not determined.
†Based on 4 highly variable genomic sequences.

showed that patients with chronic asymptomatic carriage of *T. whipplei* in fecal specimens had antibodies to *T. whipplei*; patients with WD did not exhibit or exhibited a low reaction to *T. whipplei* (*29*). The lack of antibodies for *T. whipplei* in patients points to a recent infection in nonimmune persons.

The pathogenic role played by *T. whipplei* in pneumonia is difficult to define. In the first patient (specimen no. 5), the role of *T. whipplei* in pneumonia is probable. In fact, it was the only pathogen we detected using the broad-range PCR-based assay, and it was present at a high level in an immunocompromised patient (5.10^5 copies of the genome) (Table 1). This patient had community-acquired pneumonia when admitted to the hospital. Results for the 5 other case-patients should be interpreted more cautiously. For the patients from whom specimens no. 10, 82, 86, and 183 were collected, amplification of the 16S rDNA gene showed the presence of several other bacteria usually found in oral flora. For the patients from whom specimens 10 and 82 were collected, both of whom had aspiration pneumonia, we hypothesize that *T. whipplei* was part of the oral or digestive flora that contaminated this patient with many other pathogens. It is likely that this was also true for the patients from whom specimens 86 and 183 were collected, both of whom had ventilator-associated pneumonia. For the remaining patient (specimen no. 209), the situation was more complex. In fact, we found oral and dental bacteria, 3 water bacteria, and *Pseudomonas stutzeri*, which is an environmental bacterium. The role of *T. whipplei* in this patient remained unclear.

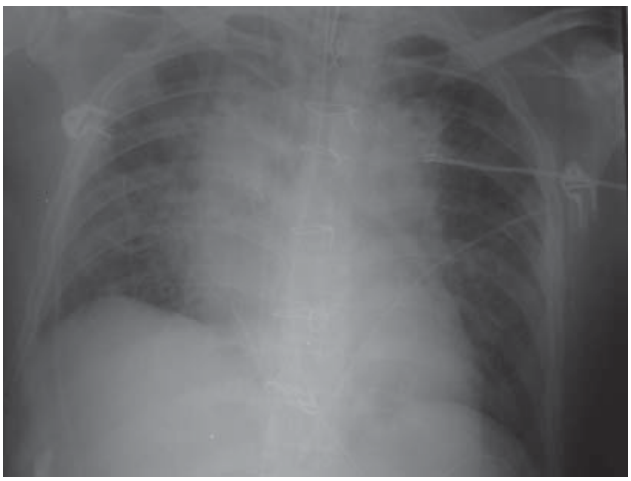

Figure. Chest radiograph of patient no. 5, who had community-acquired pneumonia associated with *Tropheryma whipplei*.

In summary, we reported a series of patients in whom *T. whipplei* DNA was found in BAL fluids. In 1 patient (specimen no. 5), the role of *T. whipplei* in pneumonia is highly convincing. For the 5 other cases, *T. whipplei* was identified, as were other oral and dental bacteria. The pathogenic role played by *T. whipplei* in the pneumonia for these 5 patients in conjunction with other bacteria is difficult to define but cannot be excluded. Our findings confirm those reported by Harris et al. (*19*), who found *T. whipplei* DNA in the sputum of a child with pneumonia. The role of *T. whipplei* in ICUs remains to be elucidated. *T. whipplei* is an ubiquitous bacterium in the digestive tract, and it has been shown that *T. whipplei* might be present in the saliva of asymptomatic persons (*2*,*30*). It is therefore probable that *T. whipplei* could contribute to the occurrence of aspiration pneumonia along with bacteria present in the oral flora. The presence of *T. whipplei* as the unique identified agent in one of the reported cases is suggestive of a real pathogenicity of this agent.

It is too early to conclude whether *T. whipplei* is an etiologic agent of aspiration or isolated pneumonia. Nevertheless, the existence of *T. whipplei* DNA in ≈3% of BAL fluid samples collected from patients with pneumonia undoubtedly raises questions about its role in the genesis of pneumonia that develops in ICU patients.

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Emergence of Increased Resistance and Extensively Drug-Resistant Tuberculosis Despite Treatment Adherence, South Africa

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We investigated the emergence and evolution of drug-resistant tuberculosis (TB) in an HIV co-infected population at a South African gold mine with a well-functioning TB control program. Of 128 patients with drug-resistant TB diagnosed during January 2003–November 2005, a total of 77 had multidrug-resistant (MDR) TB, 26 had pre–extensively drug-resistant TB (XDR TB), and 5 had XDR TB. Genotyping suggested ongoing transmission of drug-resistant TB, and contact tracing among case-patients in the largest cluster demonstrated multiple possible points of contact. Phylogenetic analysis demonstrated stepwise evolution of drug resistance, despite stringent treatment adherence. These findings suggested that existing TB control measures were inadequate to control the spread of drug-resistant TB in this HIV co-infected population. Diagnosis delay and inappropriate therapy facilitated disease transmission and drug-resistance. These data call for improved infection control measures, implementation of rapid diagnostics, enhanced active screening strategies, and pharmacokinetic studies to determine optimal dosages and treatment regimens.

The emergence of drug-resistant tuberculosis (TB) is often attributed to the failure to implement proper TB control programs and correctly manage TB cases (*1*,*2*). Consequently, >450,000 multidrug-resistant (MDR) TB cases (resistant to at least isoniazid and rifampin) are estimated to occur globally each year, of which 1%–2% occur in South Africa (*3*,*4*). A recent survey conducted by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) estimated that 7% of MDR TB samples were also extensively drug-resistant (XDR) TB (*5*), i.e., resistant to isoniazid and rifampin and to at least 1 representative of each class of the most effective second-line drugs (i.e., fluoroquinolones and the injectable drugs kanamycin, amikacin, or capreomycin). Concern over XDR TB was further heightened with the identification in 2006 of an XDR TB outbreak involving 53 cases in South Africa (*6*). This outbreak had an exceptionally high proportion of deaths among HIV co-infected case-patients and demonstrated the need for improved basic TB control measures (*7*) and enhanced infection control. Subsequently, those involved in investigating the outbreak suggested that, in the absence of drug-susceptibility testing (DST), the evolution of MDR TB and XDR TB was inevitable (*8*). A recent study in Uzbekistan showed the emergence of XDR TB while patients were being treated for MDR TB, which suggests that treatment regimens should be optimized and strategies developed for administering these regimens safely and effectively (*9*).

In 1999, WHO put forward the directly observed treatment short course (DOTS)–plus strategy, which proposed that the diagnosis and treatment of MDR TB could complement a well-functioning DOTS program and thereby con-

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trol the emergence and spread of TB (*10*). These strategies have been implemented since 2000 by the health service at a gold mine in the North West Province in South Africa. Furthermore, a policy of biannual chest radiographic screening has been instituted, which contributes to the early identification of patients with active pulmonary TB (PTB). Using these rigorous case-finding and treatment strategies, the program has been able to achieve successful treatment outcomes in >85% of new sputum smear–positive TB cases since 2001 and an average of 77.2% successful for retreatment of smear-positive cases (A.D. Calver, unpub. data). Despite this success, the incidence of drug-susceptible TB has continued to rise, an increase that reflects both the rising HIV prevalence in this community and the occupational risks specific to the mine setting such as silicosis, congregate living, and working conditions. In 2003, a marked increase (2.4×) in the number of case-patients with DR TB was noted at this gold mine. We investigated this outbreak using a molecular epidemiologic approach and clinical and epidemiologic data to identify inadequacies in the implemented DOTS-plus strategy that lead to the emergence of pre–XDR TB (MDR TB with resistance to either kanamycin or ofloxacin [*11*]) and XDR TB.

Materials and Methods

Study Population

This study was conducted at a gold mine in South Africa, January 2003–November 2005. Any employee with a new lesion detected on biannual occupational health chest radiographic screening is referred to the hospital. Similarly, all persons with self-reported suspected TB and unexplained weight loss, unexplained persistent cough for >2 weeks, and unexplained night sweats are also referred for TB investigation. A bacteriologic diagnosis of PTB was made by auramine-O fluorescent stain microscopy of 4 concentrated sputum smears and 2 TB cultures using Mycobacterial Growth Indicator Tube (MGIT) (BD Diagnostic Systems, Franklin Lakes, NJ, USA). Patients who had negative smear and culture results and chest radiographic results suggestive of PTB were monitored with repeat sputum smears. They were treated for TB only if other causes for the lesion could not be found and the patients' symptoms and radiographic results deteriorated. DST for isoniazid and rifampin was performed on all positive *Mycobacterium tuberculosis* cultures by using MGIT. Time from seeking treatment until diagnosis of drug resistance (isoniazid and rifampin) ranged from 21 to 112 days. Second-line DST was done for ethambutol, ofloxacin, and kanamycin in MGIT 960 media containing 5 μg/mL, 2 μg/mL, and 4 μg/mL, respectively. Pyrazinamide DST was carried out according to the BACTEC manual (BD Diagnostic Systems) (*12*).

Patients with bacteriologically confirmed cases of TB were treated according to WHO/International Union Against Tuberculosis and Lung Disease guidelines. They were treated within the hospital to limit community transmission until sputum smears were negative for 2 consecutive specimens collected on separate days (checked weekly). Thereafter, patients received supervised outpatient treatment, and adherence was monitored by observing the patient receive and swallow the issued daily doses. Adherence rates were reported to the mine healthcare service management and ranged from 95% to 98%.

Patients with a diagnosis of MDR TB were hospitalized and treated based on current DOTS-plus guidelines from the South African National TB control program. Treatment regimens included at least 4 drugs and were based previous treatment history and DST. Injectable drugs were stopped when sputum cultures were negative for at least 2 successive months or when side effects necessitated discontinuance. Once sputum cultures had been negative for at least 3 successive months, patients were discharged to outpatient treatment. Oral medication was continued for at least 12 months after the first negative culture, with a minimum total duration of 18 months. An outcome of cure was assigned to patients with MDR TB or XDR TB if they maintained culture conversion and completed a full course of treatment for ≥18 months. Transferred out was defined as a patient who was transferred to another healthcare facility for further TB treatment.

Infection Control

Patients with positive smear cultures were admitted to a TB ward, which is equipped with UV lights and is separated from the rest of the hospital wards by a 100-m cross-ventilated corridor. The windows in the TB ward are open throughout the year, creating good natural cross-ventilation. Although patients were advised to avoid close contact with patients from other wards, patients were not confined to this ward, and some contact may have occurred within the hospital grounds. In addition, some patients were diagnosed with PTB while they were hospitalized and were being assessed for other conditions. Such patients were subsequently transferred to the TB ward. Before 2004, patients with DR TB were hospitalized in a miniward within the TB ward used for patients with drug-susceptible disease. However, in response to the increase in the number of MDR TB patients, a separate MDR TB ward was opened in 2004. This ward is separated from the rest of the hospital by electronically locked doors for restricted entry and exit, is fitted with ceiling mounted UV air sterilizing units, and patients leaving the ward are fitted with a PF95 mask. However, patients are kept on the general TB ward until drug-resistant disease is confirmed, at which point they are transferred to the MDR TB ward.

Participant Enrollment

All mine employees and dependents with drug-resistant TB diagnosed during January 2003–November 2005 were included in this study (average number of persons covered per year: 28,943 in 2003, 25,541 in 2004, and 21,790 in 2005). Clinical and demographic data were collected retrospectively and included the following: age, sex, site of disease (PTB or extrapulmonary TB), sputum smear results, treatment category (new or retreatment cases), TB outcome, HIV status (despite extensive patient education and counseling, there are reluctance and denial issues relating to HIV testing), antiretroviral treatment (ART), place of residence within the mine area, and dates and locations of hospital stays. This study was approved by the ethics committee (internal review board) of Stellenbosch University, Tygerberg, South Africa.

Genotyping

Drug-resistant *M. tuberculosis* isolates were genotyped by insertion sequence (IS) *6110* restriction fragment-length polymorphism (RFLP) (*13*), spoligotyping (*14*), and mycobacterial interspersed repetitive unit (MIRU) typing (12-loci format) (*15*). The *katG*, *rpoB*, *pncA*, *embB,* and *gyrA* genes of the *M. tuberculosis* isolates were sequenced by using the ABI PRISM DNA sequencer (Applied Biosystems, Foster City, CA, USA) to identify nonsynonymous single nucleotide polymorphisms conferring isoniazid, rifampin, pyrazinamide, ethambutol, and ofloxacin resistance, respectively (*16*). Strains that share an identical genotype (spoligotype, IS*6110* RFLP, and MIRU type for low copy-number strains) were classified as clustered; clustered strains were considered to be part of an ongoing chain of transmission. Isolates with unique strain genotypes from new cases-patients with drug-resistant TB were considered to have primary resistance, whereas isolates with unique strain genotypes from patients undergoing retreatment were thought to have either acquired resistance during therapy or to be a reactivated a drug-resistant strain (*17*).

To elucidate the molecular evolution of drug resistance within a transmission chain, we conducted a phylogenetic analysis using DNA sequence data from isolates from the single large cluster detected through genotyping. We used 2 distinct algorithms: 1) the heuristic parsimony algorithm, and 2) the neighbor-joining distance algorithm in conjunction with sampling the original dataset with replacement to construct a series of 1,000 bootstrap replicates of the same size as the original dataset (PAUP 4.0* software version 4; Sinauer Associates, Sunderland, MA, USA) (*18*). A consensus tree was generated by using the program CONTREE (PAUP 4.0*) in combination with the majority rule formula.

Contact Tracing

Hospital and employment records were reviewed to identify potential sites of contact between patients in the largest cluster. We considered patients to have been exposed to MDR TB within the hospital setting if they been previously admitted to the hospital before their admission for diagnosis with MDR TB, during which time a patient with active MDR TB of an identical genotype had also bee also hospitalized. Patients were considered to have had work contact if they had worked the same mine shaft as a person with an MDR TB diagnosis, and to have had residential contact if they lived in the same building or group of buildings before MDR TB diagnosis.

Statistical Analysis

For univariate analyses of clustering, we used logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). We calculated p values by using the Mantel-Haenszel χ^2 method or Fisher exact test. Statistical tests were 2-sided. We used a multivariate logistic regression model to control for possible confounders. We also used a logistic regression model to estimate the OR of death among patients infected with clustered strains. Analyses were performed with STATA software version 9.0 (StataCorp LP, College Station, TX, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

During the study period, 3,003 patients with TB were notified; 1,443 (48%) had new PTB cases, 755 (25%) were being re-treated for PTB, and 805 (27%) patients had extrapulmonary TB. Of these case-patients, 70% sought treatment on their own at healthcare clinics or hospital with symptoms, while the remaining 30% were identified by active screening. Successful treatment (cure or treatment completed) was achieved in 86.5% of all TB case-patients during the study. Less than 2% of TB case-patients defaulted or had an unsuccessful treatment outcome, and 12% of TB case-patients died as a result of TB or other causes. One hundred twenty-eight (4.3%) TB case-patients had drug-resistant TB; of those, 13 (10.2%) were diagnosed with isoniazid-resistant TB, 7 (5.6%) with poly–drug-resistant TB, and 108 (84.4%) with MDR TB. Among isolates identified as MDR TB, 26 were pre–XDR TB and 5 were XDR TB.

Table 1 summarizes demographic and clinical characteristics of the cohort of 128 patients with drug-resistant TB. All of the employees had worked at the mine for at least 6 months (median 15.0 years, range 0.5–27 years) and had passed a preemployment physical examination that ruled out active TB. Among those who were HIV seropositive, 60 (70.4%) had smear-positive TB and 52 (62.0%) had a CD4 count of <200 cells/mm^3 (median 74 cells/mm^3). Fif-

ty-six (66.6%) of those seropositive for HIV were receiving HIV education from the wellness HIV clinic; 7 (8.3%) had initiated ART before being diagnosed with drug-resistant TB, and 22 (26.2%) had initiated ART after being diagnosed with MDR TB. Among those who started ART after being diagnosed with drug-resistant TB, the median time from diagnosis to initiation of ART was 172 days (range 41–1,425 days).

Outcomes were generally poor, with only 31.3% completing treatment with confirmed bacteriologic cure. Forty-five (35.2%) patients died; 7 (15.6%) of those who died did not have a confirmed TB diagnosis at time of death, 12 (26.7%) had been diagnosed with TB but were receiving standard therapy at the time of death, and 26 (57.8%) case-patients who were receiving MDR TB treatment died. Of note, >50% of the deaths were due to other AIDS-related conditions. Among those who died, median time to death from beginning of treatment was 5 months (range 1–24 months).

Combined genotype analysis of isolates from 124 of the 128 case-patients identified 61 distinct drug-resistant genotypes (online Appendix Figure, www.cdc.gov/EID/content/16/2/264-appF.htm). Fifty isolates were unique and 74 were clustered. Among the 11 clusters, the cluster size ranged from 2 to 42. We estimate that at least 63 (85.1%) of the 74 clustered isolates had primary drug resistance, assuming that each cluster was initiated by an isolate that acquired drug resistance (*19*). Among the 50 unique genotypes, 25 (50%) were cultured from new case-patients, which suggests primary drug resistance. Accordingly, we suggest that 71% of drug-resistant TB cases resulted from transmission of preexisting drug resistant strain.

Clustering was more frequent among case-patients with MDR TB than among those with monoresistant or polyresistant strains (unadjusted OR 14.20, p = 0.001; adjusted OR 14.13, p = 0.002) (Table 2). When we compared clustering among pre–XDR TB and XDR TB isolates and those with monoresistance or polyresistance, we found that these highly resistant strains were also more likely to be in a cluster than those with less resistance (unadjusted OR 27.42, p<0.001). Twenty (76.9%) of the pre–XDR TB strains and 4 (80%) of the XDR TB strains clustered with circulating MDR TB strains. Table 2 illustrates that additional risk factors for clustering were not identified in either the univariate or multivariate analysis, although patients in clusters were more likely to die than those whose

Table 1. Characteristics of mine workers with drug-resistant TB diagnosed January 2003–January 2005, South Africa*

Characteristic	No. (%) HIV+ workers	No. (%) HIV– workers	No. (%) workers with unknown HIV status	Total no. (%) workers
Total	84	7	37	128
Age (average)	43	43	42	43
Sex				
M	82 (97.6)	7 (100)	35 (94.6)	124 (96.9)
F	2 (2.4)	0	2 (5.4)	4 (3.1)
Case definition				
New	38 (45.2)	0	18 (48.6)	56 (43.7)
Retreatment†	46 (54.8)	7 (100)	19 (51.4)	72 (56.3)
Sputum smear‡				
Positive	60 (70.4)	4 (57.1)	31 (83.8)	95 (74.2)
Negative	24 (28.6)	3 (42.9)	5 (13.5)	32 (25)
Drug-resistance phenotype				
Isoniazid (mono) resistant	8 (9.5)	1 (14.3)	4 (10.8)	13 (10.1)
Poly resistant	4 (4.8)	0	3 (8.1)	7 (5.5)
MDR	72 (85.7)	6 (85.7)	30 (81.1)	108 (84.4)
Pre–XDR	22 (26.2)	3 (42.9)	1 (2.7)	26 (20.3)
XDR	2 (2.4)	1 (14.3)	2 (5.4)	5 (3.9)
Outcome				
Treatment completed or bacteriologic cure	26 (30.1)	3 (42.9)	11 (29.7)	40 (31.3)
Failed	1 (1.3)	0	0	1 (0.8)
Died	31(36.9)	2 (28.6)	12 (32.4)	45 (35.2)
Transferred out	20 (23.8)	2 (28.6)	10 (27.0))	32 (25.0)
Lost to treatment§	6 (7.1)	0	4 (10.8)	10 (7.8)
CD4 cell count, cells/mm^3¶				
<200	52 (62)	ND	5 (13.5)	57 (44.5)
>200	18 (21.4)	2 (28.6)	ND	20 (15.6)

*TB, tuberculosis; MDR, multidrug-resistant; XDR, extensively drug-resistant; ND, not determined.
†All prior TB episodes were treated at the mine.
‡1 sputum smear result missing.
§One had monoresistant TB and 9 had MDR TB.
¶CD4 counts available for 77 patients only.

isolates were not clustered (unadjusted OR 2.28, p = 0.04; adjusted OR 4.76, p = 0.007). Of note, 59% of clustered case-patients had a documented previous episode of TB, which suggests reinfection with a circulating strain (Table 2). Although higher CD4 counts were associated with less clustering (unadjusted OR 0.49, p = 0.19; adjusted OR 0.51, p = 0.28), this association did not reach significance.

Figure 1 demonstrates that most patients in the largest cluster had multiple different types of contact; 32 (76.2%) had a non–MDR TB hospitalization at the same time another patient in the cluster was admitted for MDR TB. Thirty-nine (92.9%) patients worked in a shaft in which another MDR TB patient in the cluster had worked, and 36 (85.7%) of the patients resided in the same residential unit where another MDR TB patient had lived.

Phylogenetic reconstructions of the isolates included in the largest identified a single genetically distinct progenitor MDR TB strain (Figure 2). This strain acquired resistance to pyraziamide on 2 separate occasions and both of these strains were subsequently transmitted. Thereafter, ethambutol resistance evolved independently in several different cases. Sequencing of the *gyrA* gene showed that ofloxacin resistance subsequently evolved on 6 separate occasions, resulting in 15 cases of pre–XDR TB. One of these pre–XDR TB strains then evolved to XDR TB and caused disease in a single patient (patient 27). An additional XDR TB strain evolved independently (lacking *gyrA* mutations) (patient 41) and subsequently spread to a contact (patient 141).

Discussion

Using a combination of clinical, epidemiologic, and molecular data, we showed that drug-resistance was primarily transmitted in this mine setting, thereby suggesting that the current TB control program was largely able to prevent the acquisition of drug-resistance to at least the first-line anti-TB drugs could not prevent the transmission of preexisting MDR TB in this highly vulnerable population. Similar findings have been reported for community based settings in South Africa (*21*,*22*). Our results also demonstrated that a large proportion of patients who had a previous documented episode of TB were reinfected with a circulating MDR TB strain. These findings are consistent with those from another recent study which showed

Table 2. Patient risk factors for having clustered TB isolates, January 2003–January 2005, South Africa*†

Category	Cluster status		Univariate OR	p value	Multivariate OR	p value
	Unique, n = 50	Clustered, n = 74				
Treatment history						
Re-treatment	25	44				
New case	25	30	1.47	0.29	0.69	0.50
Sex						
M	49	71				
F	1	3	1.27	0.52	NI	
Age, y						
<45	33	51				
≥45	17	23	0.87	0.73	NI	
HIV status						
Negative	4	3				
Positive	29	53	2.43	0.26	2.33	0.33
Sputum smear						
Negative	14	19				
Positive	36	55	1.12	0.77	NC	
MDR TB						
Mono or poly resistant	16	2				
INH and RIF resistant	34	72	14.2	0.001	14.13	0.002
MDR plus						
Mono or poly resistant	16	2				
Pre–XDR TB/XDR TB	7	24	27.42	<0.001	NC	
Died						
No	38	42				
Yes	12	32	2.28	0.04	4.76‡	0.007‡
CD4 count, cells/mm^3						
<200	15	42				
>200	8	11	0.49	0.19	0.51§	0.28

*TB, tuberculosis; OR, odds ratio; NI, variables not included in final logistic regression model based on model selection criteria; NC, variables a priori not considered in logistic regression model; MDR, multidrug-resistant; XDR, extensively drug-resistant; INH, isoniazid; RIF, rifampin.
†Logistic regression performed on 71 observations for which HIV and CD4 available, model adjusted for treatment history, HIV, CD4, and MDR.
‡Logistic regression on probability of death given clustering, adjusting for HIV, MDR-plus, and age.
§CD4 counts available for 77 patients only.

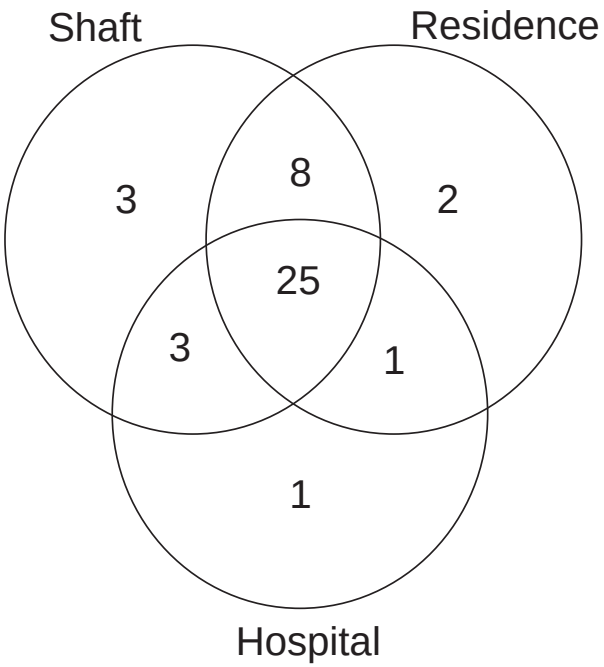

Figure 1. Venn diagram of number of potential contacts by type among patients in the largest multidrug-resistant tuberculosis (MDR TB) cluster, South Africa, 2003–2005. Each circle represents potential places of contact: shaft, mine shaft (work); residence, place of residence; hospital, hospitalization at the same time as another MDR TB case-patient.

that patients in China with drug-resistant TB who had previously received treatment were frequently infected with a clustered (i.e., recently transmitted) strain (*23*). In previous work, we demonstrated that a prior episode of TB may increase the risk of a subsequent episode of disease through reinfection, even in a population with a low HIV prevalence (*24*). This heightened susceptibility may be especially severe in HIV co-infected patients, given previous concerns that TB may accelerate immune suppression (*25*).

In this study, a large proportion of the HIV-infected TB patients had smear-positive TB, despite the fact that many had CD4 counts <200 cells/mm^3. These data are consistent with previous findings that suggest that 35% of TB cases in HIV-infected persons are smear negative (*26*). Most notably, this finding demonstrated that in this high risk environment, HIV co-infected patients can transmit TB to close and susceptible contacts, even in the setting of a vigorous TB control program. Of note, we found that active case finding by biannual chest radiographic screening identified only 30% of TB cases

Our phylogenetic reconstruction of the largest cluster of cases demonstrated sequential acquisition of resistance-causing mutations. We believe that the evolution of resistance to ethambutol and pyrazinamide represents the further amplification of drug resistance in the context of patients with undiagnosed MDR TB initially being given standard therapy (*27*). An MDR TB case-patient with a strain resistant to isoniazid, rifampin, ethambutol, and pyrazinamide could then spread disease to persons who were co-hospitalized for drug-susceptible TB or illnesses other than TB. Disease may develop in these persons, and they can then spread MDR TB to their contacts at their place of work or residence, thereby unintentionally perpetuating the drug-

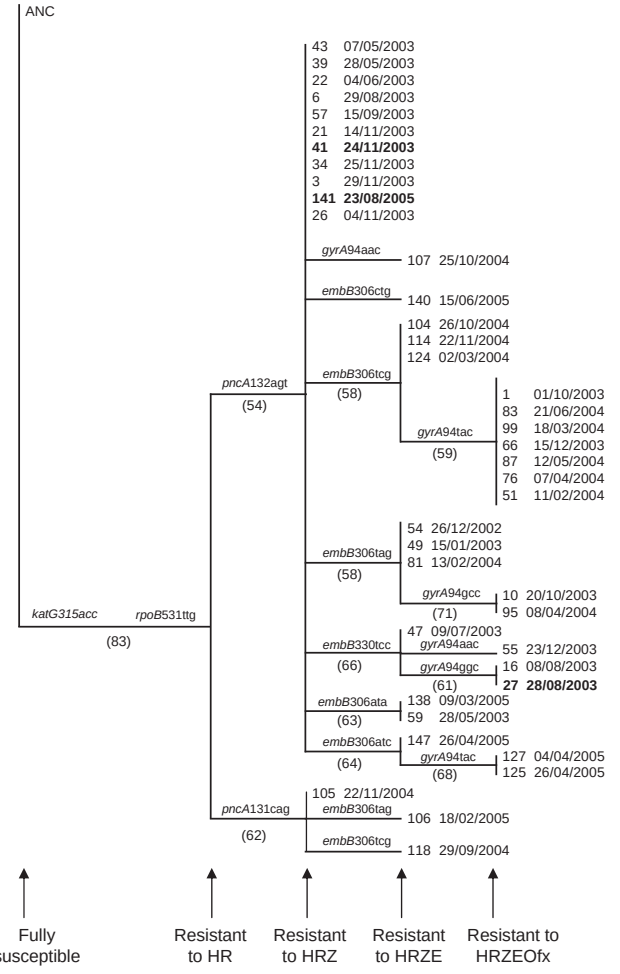

Figure 2. Phylogenetic history of the largest multidrug-resistant tuberculosis (MDR TB) cluster, South Africa, 2003–2005. Genetic data from isolates from 40 of the 42 case-patients were analyzed. The phylogenetic tree was constructed by using the neighbor joining algorithm (PAUP 4.0*; Sinauer Associates, Sunderland, MA, USA) and was rooted to the H37Rv wild-type DNA sequence (ANC) (*20*). The gene and the codon conferring resistance are indicated at the internal node where they occurred. Bootstrap values are shown in brackets at the internal nodes. The sequential evolution of resistance to HRZE and Ofx is indicated. The date of MDR TB diagnosis follows each case number. The 3 XDR TB cases are indicated in **boldface**. H, isoniazid, R, rifampin, E, ethambutol, Z, pyrazinamide, Ofx, ofloxacin.

resistant TB outbreak. We believe that this observation is not unique to this setting (*12*,*28*–*32*).

Our phylogenetic analysis also indicated that ofloxacin resistance emerged on many occasions after acquisition of resistance to first-line drugs. Although these data suggest that treatment of MDR TB patients with second-line drugs resulted in the evolution of ofloxacin resistance, the mechanisms by which patients acquired this resistance, despite excellent adherence to MDR TB treatment, remain unclear. This stresses the need for pharmacokinetic studies to optimize dosages and treatment regimens for MDR TB in HIV co-infected patients and HIV uninfected patients.

In conclusion, we recommend that DOTS and DOTS-Plus TB control programs (*33*) should be integrated with well functioning HIV management programs to ensure that ARVs are widely administered to limit susceptibility to TB disease. Furthermore, additional intervention measures are required to identify infectious cases. Such measures should include, increasing public awareness of TB symptoms, active screening of all patients making contact with the health care services and more aggressive case finding. The high proportion of smear positive cases with drug-resistant TB suggests that more frequent sputum smear examinations may allow for the early identification these infectious cases. In addition, more frequent culture based diagnosis may identify cases before they become infectious. This study also emphasizes the importance of the development and implementation of rapid DST diagnostics to minimize the delay in detecting MDR TB and the risk of inadvertently placing the patient on a regimen that could lead to the amplification of drug-resistance (*34*). Rapid DST diagnostics may help to prevent nosocomial spread of MDR TB since patients could be rapidly identified and isolated from others (*26*). Our finding that a large proportion of patients in the largest cluster were hospitalized at the same time, raises the possibility that transmission was nosocomial. This may be curtailed by more rigorous infection control measures. We recommend that adequate infection control measures should be implemented in all hospital departments and gathering places to prevent nosocomial infections.

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Associations between *Mycobacterium tuberculosis* Strains and Phenotypes

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To inform development of tuberculosis (TB) control strategies, we characterized a total of 2,261 *Mycobacterium tuberculosis* complex isolates by using multiple phenotypic and molecular markers, including polymorphisms in repetitive sequences (spoligotyping and variable-number tandem repeats [VNTRs]) and large sequence and single-nucleotide polymorphisms. The Beijing family was strongly associated with multidrug resistance (p = 0.0001), and VNTR allelic variants showed strong associations with spoligotyping families: ≥5 copies at exact tandem repeat (ETR) A, ≥2 at mycobacterial interspersed repetitive unit 24, and ≥3 at ETR-B associated with the East African–Indian and *M. bovis* strains. All *M. tuberculosis* isolates were differentiated into 4 major lineages, and a maximum parsimony tree was constructed suggesting a more complex phylogeny for *M. africanum*. These findings can be used as a model of pathogen global diversity.

Tuberculosis (TB), caused by bacteria of the *Mycobacterium tuberculosis* complex (MTBC), remains a global threat to human health, which causes an estimated 2 million deaths annually (*1*). No horizontal gene transfer has been reported in MTBC, and the genome is more highly conserved than other pathogenic bacteria (*2*). Nevertheless, genotyping tools have recently identified several polymorphisms in the MTBC genome that have provided insight into its evolution. Three major groups of MTBC genome alterations have been reported: single nucleotide polymorphisms (SNPs), large sequence polymorphisms (LSPs), and polymorphisms within repetitive sequences such as variable number tandem repeats (VNTRs). The first 2 groups mark irreversible genetic events and can be used to construct phylogenies for *M. tuberculosis* (*2*–*6*). An association between geographic region and *M. tuberculosis* families, defined by specific polymorphisms, has been demonstrated. This geographic structuring producing genetically, and perhaps phenotypically, distinct MTBC populations may contribute to differences in clinical features such as severity of disease or prevalence of extrapulmonary disease (*6*–*8*) and should be considered during the development of new drugs and vaccines.

Sreevatsan et al. divided MTBC strains into 3 principal genetic groups (PGG1–PGG3) based on SNPs in codon 463 of *katG* and codon 95 of *gyrA* (*2*). More recently, on the basis of polymorphisms in the *oxyR, katG,* and *rpoB* genes, strains have been divided into 5 lineages (I–IV and *M. bovis*); lineages I, III, and IV represent subgroups within PGG1, and lineage II corresponds to PGG 2 and 3 (*7*). By combining these markers with LSPs RD239, RD105, RD750, RD711, and RD702, a small 7bp deletion in the *pks15/1* gene and other SNPs, Gagneaux and Small were able to confirm these *M. tuberculosis* lineages and 2 lineages of *M. africanum* (*6*). The deletions RD9 and TbD1 are useful phylogenetic markers for other members of MTBC complex and ancestral *M. tuberculosis* strains (*3*). The loss and acquisition of repeats or spacers in the direct repeats region (*9*) does not appear to limit their value in biogeographic and phylogenetic studies (*10*,*11*).

Genotypic variation of MTBC strains at various geographic settings and significant associations between certain allelic variants at VNTR loci, MTBC lineages, and spoligotyping families have been reported (*7*,*12*–*15*). However, most studies used single genotyping methods on small populations or convenience samples. Population-based studies

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have focused primarily on areas of low- to middle-TB incidence, and it is unclear whether the results are universally applicable (*16*–*18*). Larger population-based studies on geographically diverse populations are needed to establish the phylogenetic, epidemiologic, and clinical relevance of such associations.

London accounts for nearly half of all TB cases in the United Kingdom (≈3,300 cases in 2006; incidence rate 44.8/100,000). Because 75% of these TB patients were born abroad (*19*), (Health Protection Agency update; www.hpa.org.uk), and clinical signs of disease develop within 2 to 3 years of arrival, we believe that the multicultural and diverse community in London provides a unique setting for studying the global biodiversity of MTBC. We aimed to establish whether MTBC isolates circulating in the London population are a useful model of global diversity, to determine the phylogenetic relevance of polymorphisms in repetitive regions of the MTBC genome, especially for *M. africanum* and its position in TB evolution, and to investigate associations between lineage and phenotype.

Materials and Methods

Study Design and Bacterial Isolates

One isolate from each of the 2,261 MTBC culture-positive patients was included in this prospectively designed population study. These isolates were collected from patients in all 30 London National Health Service hospitals between April 1, 2005, and March 31, 2006. Demographic data, including gender, date of birth, and country of birth were assigned to world regions according to an existing United Nations classification (*20*).

Identification

Cultures were identified by using standard phenotypic identification tests (*21*) and molecular methods (Genotype Mycobacterium CM, AS, and MTBC kits; Hain Lifescience GmbH, Nehren, Germany) and the INNO LiPA Rif TB assay (Innogenetics, Ghent, Belgium) performed as recommended by the manufacturer. DNA was extracted from cultures using chloroform extraction as described (*22*). Isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide, and ciprofloxacin susceptibilities were determined by using the resistance ratio method (*21*).

Genotyping

All extracts were typed by using automated 15 mycobacterial interspersed repetitive unit–VNTR (MIRU-VNTR) fragment analysis (*23*–*26*). Clustered isolates were further genotyped by using an extended panel of 7 hypervariable VNTR loci (*27*). Data were exported to BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) for cluster analysis.

Spoligotyping was performed according to the manufacturer's instructions (Isogen Lifescience, IJsselstein, the Netherlands) (*9*). Images were digitized and entered into BioNumerics software by using the BNIMA module (Applied Maths). Spoligotypes were assigned to families and subfamilies by using the online tools at http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html (*10*). We have used the established spoligotyping families Beijing, Central Asian (CAS), East African–Indian (EAI), and *M. bovis* as lineage designations, as well as European American (EuroAm) (*13*,*28*) for the *M. tuberculosis* lineage, which includes the X, T, LAM, S, and Haarlem families.

Other Methods

Detection of TbD1 and RD9 (*3*,*13*) was conducted by PCR fragment analysis (*3*). Reverse hybridization methods were used to analyze the 4 lineage-defining SNPs in 3 genes (*oxyR*^{C37T}, *katG*^{C87A}, *rpoB*^{T2646G}, and *rpoB*^{C3243T}) reported by Baker et al. (*7*) for selected isolates (n = 259) (*12*) and mutations in *katG*, *inhA*, and *rpoB* genes associated with drug resistance (*22*).

Data were analyzed by using Excel, BioNumerics (Applied Maths), SPSS 12.0 (SPSS Inc, Chicago, IL, USA) software and online interactive statistical tools (www.quantitativeskills.com/sisa/). Categorical variables were analyzed by using relative risks (RRs), odds ratios (ORs), and the χ^2 test. Discrimination power of genotyping methods was assessed using the Hunter-Gaston index (*29*).

Results

Diversity within the Study Population

We studied 2,261 isolates, representing 95.7% of all the bacteriologically confirmed TB cases reported in London from April 1, 2005, through March 31, 2006. Using routine phenotypic and genotypic methods, we identified 99.1% (2,241) as MTBC; the remaining 20 were too heavily contaminated for analysis.

Spoligotypes were generated for 98.8% (2,233) of the isolates; 656 types were identified, of which 458 were unique and 198 were shared by groups of 2–221 isolates. Isolates were assigned to families and subfamilies on the basis of their spoligotype by using the online tools at http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html. All but 4 spoligotypes were assigned to ≥1 of 36 groups; 88.4% of isolates were assigned to a single spoligotyping family or subfamily. The remaining 11.6% were assigned to 2 families, albeit with given probabilities of <0.9. All the main spoligofamilies seen globally were represented within this population (Table 1).

Isolates were cultured from a variety of body sites; 57% were of pulmonary origin. Where known, 60% of isolates were cultured from male patients and 40% from female

patients; median age was 33 years. The COB was available for 1,381 (61.0%) patients; 1,157 (83.8%) were born in 89 countries outside the United Kingdom (online Appendix Table, www.cdc.gov/EID/content/16/2/272-app-T.htm). The population included representatives from all regions of the world (*20*).

VNTR Data as Phylogenic Markers

The 22 MIRU-VNTR genotypes, generated for 2,261 isolates, resulted in 1,434 VNTR types representing the minimum number of independent strains within this population. Each type was designated an MTBC lineage on the basis of the VNTR types (*12*) (Figure 1). Where these lineages were ambiguous (n = 49), discordant to those suggested by spoligotype (n = 58), or not defined (n = 210), SNP analysis was performed to resolve these differences (n = 317). In all cases, the SNP analysis resolved the ambiguous VNTR lineage calling as 1 of the alternatives producing the ambiguity. The SNP-defined lineage of strains discordant between the spoligotype and VNTR agreed with the VNTR call in 74.0% of cases. Finally, among the strains for which the VNTR was unable to define any lineage, there was 94.0% agreement between the SNP and spoligotype-defined lineage (Table 1). All strains identified as *M. africanum* were placed in the nondefined group and had the SNP-1 genotype.

Spoligotyping gave a lineage that was confirmed by an independent marker (VNTR or SNP) in 96.3% of isolates. VNTR gave an unambiguous lineage in 77.9% of strains; of these, 99% were confirmed by an independent marker (spoligo or SNP). Allelic variants were sought at each VNTR locus that best described each spoligofamily; those giving the highest sensitivities and specificities are shown in Table 2. The highest sensitivities were seen in the LAM 1, LAM 10, and Beijing families, which suggests their highly clonal and homogeneous nature. Several allelic variants showed strong associations with spoligo families, with ≥5 copies at ETR-A, ≥2 copies at MIRU24, and ≥3 copies at ETR-B associated with EAI and *M. bovis* (RR 2.99, 95% confidence interval [CI] 2.51–3.56; RR 6.29, 95% CI 4.87–8.12; and RR 3.21, 95% CI 2.63–3.93, respectively), ≥3 copies at MIRU4 and 2 copies at MIRU26 with EAI (RR 2.31, 95% CI 1.98–2.70; and RR 12.8, 95% CI 8.41–17.90, respectively), and 4 copies at MIRU23 with *M. africanum* and *M. bovis* (RR 220.3, 95% CI 82.07–591.50).

The presence of 2 copies at MIRU24 appears to be a good marker for EAI *M. tuberculosis* and non–*M. tuberculosis* members of the MTBC. This marker (number of copies in the locus MIRU24) was investigated in this population by using the occurrence of the deletions RD9 and TbD1, which have previously been used as markers to distinguish these groups (Table 3). All 41 isolates identified as *M. africanum* by spoligotype were also analyzed in this

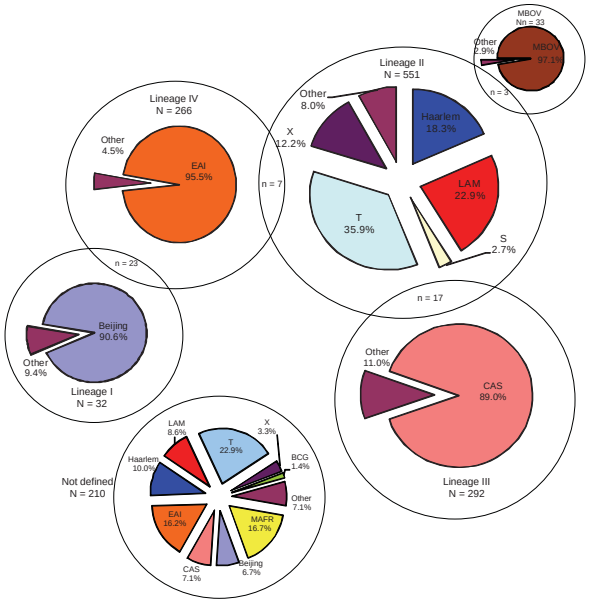

Figure 1. *Mycobacterium tuberculosis* complex lineages as determined by Gagneux et al. (*6*) and Baker et al. (*7*) defined by mycobacterial interspersed repetitive unit codes. MBOV, *M. bovis*; LAM, Latin American; CAS, Central Asian; EAI, East African–Indian; BCG, bacillus Calmette-Guérin; MAFR, *M. africanum*. The X, T, LAM, S, and Haarlem families are European American types.

manner, 11 of which contained a single copy of MIRU24; 296 *M. tuberculosis* isolates containing single and double copies of MIRU24 were analyzed as controls.

The deletion TbD1 was present in all EuroAm, CAS, and Beijing strains examined as well as some other *M. tuberculosis* isolates and absent from all *M. africanum* isolates. The deletion RD9 was present in all *M. bovis* strains as well as some EAI and most *M. africanum* strains but absent from all other strains. Both deletions were absent from most EAI and some *M. africanum* strains (Table 3). Absence of RD9 deletion and 2 copies in MIRU24 was strongly associated with EAI spoligotype (RR 15.1, 95% CI 9.49–23.89). MTBC strains with the RD9 intact and 2 copies in MIRU24 included both *M. bovis* and *M. africanum* spoligotypes, whereas strains with the RD9 intact and 1 copy in MIRU24 formed a specific group of *M. africanum* originating presumably from the Indian subcontinent. Using this data, and the SNP 1-MB and the MIRU24 enumeration data, we constructed a maximum-parsimony tree as shown in Figure 2.

Associations between Phylogenic Groups and Phenotype

Strong associations were seen between patient's country of origin and the spoligo family of the isolate (online Appendix Table): CAS and EAI families dominated in

Table 1. Analysis of associations between *Mycobacterium tuberculosis* phylogenetic lineages defined by SNP analysis and spoligotyping families in the group of isolates not classified using VNTR codes, UK*

	Lineages (*6,7*) and relevant MIRU codes (*12*)				
	M. tuberculosis				*M. bovis*; 10–2, 40–2, C–5
Spoligotypes	I/East Asian; 39–3, A–4, C–4	II/European American; 16–1,2,3, 39–2, B–1,2	III/EAI; 23–5, C–2	IV/Indo-Oceanic; 24–2, 26–2	
H37Rv, n = 2	0	2	0	0	0
Beijing, n = 13	**13**	0	0	0	0
LAM, n = 17	0	**17**	0	0	0
T, n = 53	1 600740007764671†	**51**	0	1 777200007403371†	0
Haarlem, n = 21	0	**20**	0	1 777777774000731†	0
EAI, n = 61	2 777777770003331† 477777377413771†	1 777734000000031†	0	**58**	0
CAS, n = 18	0	0	**18**	0	0
X, n = 9	0	**9**	0	0	0
S, n = 2	0	**2**	0	0	0
Family 33, n = 4	1	1	0	**2**	0
Family 35, n = 7	**7**	0	0	0	0
Family 36, n = 5	0	**5**	0	0	0
M. bovis BCG, n = 4	0	0	0	0	**4**
M. africanum, n = 35	**34**	1 710044706302261*	0	0	0

*SNP, single nucleotide polymorphism; VNTR, variable number tandem repeat; MIRU, mycobacterial interspersed repetitive unit; EAI, East African–Indian; LAM Latin American; CAS, Central Asian. European American includes the X, T, LAM, S, and Haarlem families.
†Octal codes indicate spoligotyping patterns for isolates with disagreements between SNP- and spoligotype-defined lineages. Dominant families within each lineage are in **boldface**.

patients born on ISC (RR 2.4, 95% CI 2.02–2.74) as did Beijing and EAI families in patients born in Southeast Asia (RR 4.8, 95% CI 2.70–8.54). EAI families were seen in 80.4% of isolates from patients born in East Africa and the ISC. The *M. africanum* family dominated in patients born in West Africa (RR 3.67, 95% CI 1.52–6.50). In contrast, LAM and Haarlem isolates were infrequently seen in patients born on the ISC (4.5% and 5.4%) and Southeast Asia (3.4% and 6.9%). T family isolates, one of the genetic groups determined by spoligotyping, were distributed evenly across all regions except Southeast Asia, where they were infrequently seen. No association between lineage or spoligo family and pulmonary versus extrapulmonary site was seen in the present study.

Susceptibility to rifampin, isoniazid, ethambutol, streptomycin, and pyrazinamide was evident for 98.9% (2,236) of the isolates. Of these isolates, 84.3% were sensitive to all, 8.2% were isoniazid resistant, 5.4% streptomycin resistant, 1.5% rifampin resistant, 0.7% ethambutol resistant, 0.5% pyrazinamide resistant, and 1.2% multidrug resistant.

Associations between spoligotype families and drug resistance of MTBC strains were analyzed by determining the minimum number of independent clones and the minimum number of resistance acquisition events within this population. VNTR15 cluster analysis was performed on all isolates (n = 2,261) to identify a single representative of each unique genotype. This analysis resulted in 1,166 unique types.

When isolates shared a genotype but differed in susceptibility to a given drug, resistant and sensitive isolates were analyzed because the resistant isolate must have undergone a genetic event and acquired a unique genotype. When genotypes for loci associated with isoniazid and rifampin resistance had been determined and >1 type was present in a cluster, 1 of each type was included. Where members of a cluster and its nearest neighbor were resistant, this was considered as a single acquisition event and only a single member was included. The resulting numbers divided between spoligotype families are shown in Table 4.

The *M. bovis* BCG family was associated with pyrazinamide ($p<0.0001$) and ethambutol resistance ($p = 0.0009$). Beijing family strains were associated with multidrug resistance ($p = 0.0001$), isoniazid ($p = 0.0019$), and rifampin ($p = 0.0027$) resistance. Associations were seen between streptomycin resistance and the Beijing family ($p = 0.0008$) and between pyrazinamide ($p = 0.0079$) and streptomycin ($p = 0.008$) resistance and the LAM1 family.

Discussion

Several approaches have been used to study the global diversity of MTBC. One approach is to construct a global sample of isolates from reference collections around the world (*19*,*30*,*31*). In this instance, the degree of confidence as to geographic origin of an isolate is high, but bias occurs 1) where variety is limited to sites with which investigators

Table 2. Associations between *Mycobacterium tuberculosis* MIRU15 profiles and spoligotyping families and subfamilies, UK*

Spoligotype families	MIRU15 allelic variants															Se, %	Sp, %
	2	4	10	16	20	23	24	26	27	31	39	40	A	B	C		
Beijing	2	2	2; 3	2–4	2	5; 6	1	5–8	1–3	5	2–4	1–4	3; 4	2	4	80.0	99.9
CAS	2	2	Any	3-5	2	5	1	Any	3	4; 5	2; 3	1–4	3; 4	2	2	72.6	98.3
EAI1	2	Any	2-6	1–4	2	5	1	4–6	3	3; 5	2; 3	<5	2–4	2	2–4	77.0	85.4
EAI2	2	>3	4; 5	2; 3	2	6	2	2–4	3; 4	3–5	3; 4	2; 3	4; 6	>2	4	73.3	99.9
EAI3	2	>2	3; 4	1–5	2	6–8	2	2	3	4–6	2; 3	Any	>4	1	3; 4	77.8	99.9
EAI4	2	2–6	4	2; 3	2	5; 6	2	2	1; 3	Any	1; 3	2; 3	>6	2–4	4	58.8	98.8
EAI5	2	1–9	3–6	2–4	2	>3	1–3	2	1–3	2–7	1–3	1–4	>4	2–7	2–4	75.0	98.3
MAF	2	2; 3	4–7	Any	2	4	1; 2	3–5	2–4	Any	2	1; 2	>3	2–4	4; 5	63.4	99.9
MBOV	2	1–3	2	3	2	4	2	5	2; 3	3	2	2	5	5–7	5	72.1	99.9
Haarlem1	2	2	2–6	2–4	1; 2	3; 5	1	4–7	3	3	2	2–5	2; 3	1; 2	3–5	66.7	89.2
Haarlem2	2	2	4; 5	1–3	1; 2	3–6	1	4; 5	1–3	3	2	1–4	2; 3	1; 2	3; 4	71.0	91.5
Haarlem3	2	2	2–6	1–4	1; 2	3; 5	1	4–6	3	2; 3	2	1–4	2–4	1; 2	2; 3	52.9	94.6
LAM1	2	2	3; 4	2	2	6	1	5; 6	2; 3	3	2	1	2	2	4	80.6	99.9
LAM10	2	2	2–4	2–4	1; 2	5	1	3–5	3	3	2	1–4	2–4	2	4	87.4	92.4
LAM3	2	2	4	2; 3	2	5; 6	1	4; 5	3	3	2	3	1; 2	2	2; 4	78.4	99.9
LAM5†	2	2	4	3	2	5	1	9	2	4	2	4	2	2	4	100.0	100.0
LAM7†	2	2	4	3	2	6	1	3	3	3	2	3	2	2	4	50.0	100.0
LAM8	2	2	2–5	1; 3	2	5 6	1	4; 5	1–3	3	2	1	2	1	4	66.2	99.4
LAM9	1; 2	2	2–4	1–3	1; 2	5–8	1	4–6	2; 3	2–4	2	Any	1–4	1; 2	2–6	72.7	77.1
S	2; 3	2; 3	3	2; 3	1–3	5; 6	1	4–6	3	2; 3	2	2; 4	4	2	4	44.4	99.8
T1	2	2; 3	Any	Any	2	5; 6	1	<7	2; 3	2–4	2	Any	2–4	2	2–5	54.5	85.8
T2†	2	2	3; 5	3	2	5; 6	1	5	3	2; 3	2	1; 3	2; 3	2	3–5	100.0	98.3
T3	2	2	3–5	1–3	1; 2	5; 6	1	1; 5	3	3	2	2–5	3	2	4	60.5	95.7
T4	1; 2	2–4	2–4	3	1; 2	5; 6	0–2	4–5	2; 3	2–4	2	2–4	2; 4	1; 2	2–5	46.2	88.3
X1	2	2	3–6	3	1; 2	5; 6	1	1–7	3	2–4	2	2–7	3; 4	1; 2	2–5	61.4	92.3
X2	1; 2	1; 2	4	3	2	5	1	4–8	3; 4	2; 3	2	1–4	2; 3	2	2; 4	79.5	99.3
X3	2	2	3; 4	2; 3	2	5	1	4; 5	3	2; 3	2	2–5	3	2	3	66.7	99.0

*MIRU, mycobacterial interspersed repetitive unit; Se, sensitivity; Sp, specificity; CAS, Central Asian; EAI, East African–Indian; MAF, *M. africanum*; MBOV, *M. bovis*; LAM, Latin American. The X, T, LAM, S, and Haarlem families are European American types. Any means any family or subfamily. Only strains with no secondary assignations to spoligotype groups were used for calculating associations.
†Due to a small number of isolates in these families, Se and Sp values are calculated for illustrative purposes only.

have contact and 2) sites with high TB transmission, which often lack adequate facilities for bacteriologic culture. A second approach is to study isolates derived from a population at a single geographic location whose members have diverse geographic origins throughout the world (*19*,*30*,*31*). In this instance, where country of birth data are used to indicate the geographic origin of an isolate, the degree of confidence in this data may be lower, but MTBC isolates can be sampled at a wider range of geographic locations particularly from high TB incidence areas that have poor bacteriologic isolation facilities. Furthermore, additional data such as antimicrobial drug susceptibility and site of infection, useful for association studies, are retained and the quality of the data is ensured.

London is a cosmopolitan city where up to 30% of the population is foreign born (www.neighbourhood.statistics.gov.uk), among whom 75% of TB cases are seen (*19*; HPA update, www.hpa.org.uk); a similar situation has been reported in New York and Paris (*19*,*30*,*31*), although London TB notification rates (44.8 cases/100,000 population in 2006) are generally higher than those for other high-income cities. We believe that London provides a suitable setting for studying global MTBC diversity because our study shows that TB patients came from 89 different countries

Table 3. Association between *Mycobacterium tuberculosis* spoligotypes, deletions, and allelic variants in the locus MIRU24, UK*

Deletion mapping and VNTR typing results	Spoligotype families			
			M. tuberculosis	
	Mycobacterium bovis, n = 14	*M. africanum*, n = 41	EAI, n = 241	Other, n = 55
TbD1+	14	41	239	19
TbD1–	0	0	2	36
RD9+	0	1	235	46
RD9–	14	40	6	9
MIRU24≥2	14	30	240	24
MIRU24≤1	0	11	1	31

*MIRU, mycobacterial interspersed repetitive unit; EAI, East African–Indian; VNTR, variable number tandem repeat.

Table 4. Minimum number of unique types seen within each *Mycobacterium tuberculosis* spoligotype family, by resistance or susceptibility to 5 antimicrobial drugs, United Kingdom*

Spoligotype family	No. types, by drug resistance or drug susceptibility											
	STR-R	STR-S	INH-R	INH-S	ETH-R	ETH-S	RIF-R	RIF-S	PZA-R	PZA-S	MDR+	MDR–
Beijing	10	40	13	38	3	43	5	43	1	45	5	45
CAS	14	202	30	198	1	206	3	206	2	206	3	206
EAI	8	244	23	234	3	247	4	247	1	248	4	247
European American	45	451	59	441	5	475	19	467	6	474	10	470
M. bovis BCG	0	6	2	4	1	5	1	5	2	3	1	5
Family 33–36	5	39	4	41	1	43	1	43	0	44	1	44
M. africanum	2	22	0	22	0	22	0	22	0	22	0	22

*STR, streptomycin; R, resistant; S, susceptible; INH, isoniazid; ETH, erythromycin; RIF, rifampin; PZA, pyrazinamide; MDR, multidrug-resistant; CAS, Central Asian; EAI, East African–Indian; BCG, bacillus Calmette-Guérin.

of origin, representing all regions of the world (*20*), including areas that the World Health Organization has defined as having a high incidence of TB. The bacterial diversity within this population is shown by the presence of all the main spoligofamilies, although not all lineages are equally represented. Our study shows a disproportionate representation of patients from different regions; relatively small numbers were from the Americas.

Recent advances in molecular genotyping and comparative genomics have demonstrated that the level of genetic variation in the MTBC may have been substantially underestimated. Rapidly evolving genomic regions such as VNTR and the direct repeat region have been exploited for epidemiologic studies, whereas irreversible events recorded by SNPs and LSPs are of phylogenetic value (*3*,*5*–*7*). Associations between polymorphisms in rapidly evolving genomic regions (VNTR or direct repeat region) and the SNP and LSP markers have been described (*6*,*12*,*13*,*28*,*32*–*34*). If the nature of these relationships could be clearly defined, large studies could be performed by investigating databases containing routine VNTR data.

Where lineages indicated by SNP and LSP analysis are congruent with spoligotype family names, we have retained these (as for CAS, EAI, Beijing, *M. africanum* and *M. bovis*); for the lineages containing LAM, Haarlem, X,T, and S spoligo families, we have used the lineage designation EuroAm as suggested elsewhere (*6*). We have previously reported 10 VNTR loci (ETR A,B,C; MIRU10,16,23, 24, 26,39,40) (*12*) capable of differentiating the MTBC into 4 lineages (I–IV) and *M. bovis* (*7*).

VNTR analysis showed that 1,174 (81.9%) of 1,434 independent strains could be grouped unambiguously into 5 lineages. When the remainder were grouped by using the SNP analysis, a good correlation was seen between lineage and spoligotype family or group of families (Figures 1, 2).

Discrepancies between lineage and spoligo family mainly resulted from limitations imposed by the family designation software, choice of genetic targets analyzed, or overlapping rules defining some spoligo families. Strains belonging to families 33–36 and EAI 1 appeared in multiple lineages. These spoligotype families were designated as low probability, which suggests that the model spoligotype was detecting unrelated events in different families. In rare cases, discrepancies will be seen where genetic events converge to give identical types in unrelated strains. In the present study this can be seen when multiple lineages are indicated by VNTR or spoligotypes.

Discrepancies will also occur where the VNTR/SNP system fails to distinguish between spoligotype families. The most striking of these are the strains identified as *M. africanum* by spoligotype but as the Beijing lineage because of the presence of SNP1. We resolved this problem by constructing a maximum-parsimony tree (Figure 2) using the 5 SNP, LSP, and MIRU24 repeat numbers. The

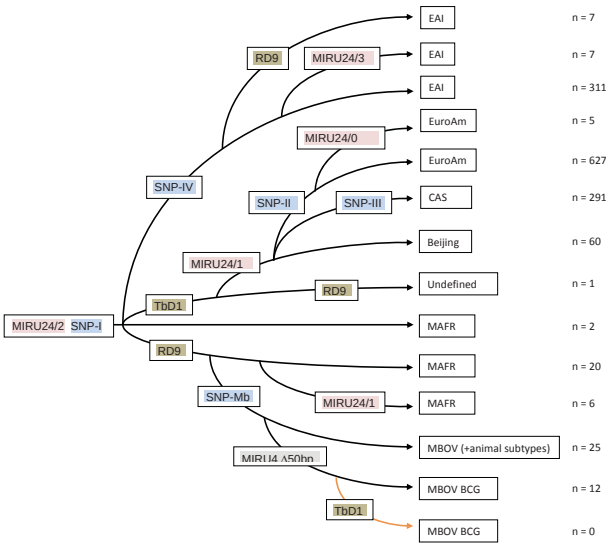

Figure 2. Maximum-parsimony tree constructed based on 3 independent sets of markers: large sequence polymorphisms (LSPs), single nucleotide polymorphisms (SNPs), and number of repeats in the locus 24 using the following assumptions: 1) SNPs are irreversible unique events; 2) LSPs are irreversible rare events; 3) spoligotypes are not produced by convergent events; and 4) variable number tandem repeat (VNTR) loci can both acquire and lose repeats. EAI, East African–Indian; MIRU, mycobacterial interspersed repetitive unit code; EuroAm, European American; CAS, Central Asian; MBOV, *M. bovis*; MAFR, *M. africanum*; BCG, bacillus Calmette-Guérin. The X, T, LAM, S, and Haarlem families are European American types.

MIRU24 repeat numbers appear to play a phylogenetic role, as shown in this study (Table 3) and previous studies (*13*,*15*) in which ≥2 repeats are markers for EAI2–EAI5 (but not EAI1), *M. tuberculosis,* and *M. bovis* strains. In its construction, we made the assumptions that SNPs mark irreversible unique events and that VNTR loci can acquire and lose repeats. A BCG strain isolated from a patient from London (not included in this study) contained the TbD1 deletion, demonstrating clearly that these deletion events are not unique. Therefore, the assumption that LSPs are infrequent irreversible events was made. The strains in this study are of human origin and therefore are mainly *M. tuberculosis* and *M. africanum*, hence the focus of the phylogenetic scenario. The tree shown here is concordant with previous scenarios (*3*,*6*) differing only in the diversity seen in strains identified as *M. africanum*. All these strains contained SNP1 and were identified on the basis of the loss of spoliogotype spacers 8, 9, and 39 but contained either 1 or 2 copies of MIRU24 and the presence and absence of RD9, resulting in 3 types. The absence of the TBD1 deletion distinguishes *M. africanum* strains from Beijing strains.

EAI strains may represent the ancestral MTBC type (*6*,*15*). The data presented here suggest that *M. africanum* competes for this distinction. The types containing 2 copies of MIRU24, with and without RD9 originate exclusively from West Africa, suggest that these may be indigenous to this region. *M. africanum* species have traditionally been phenotypically subdivided into 2 subgroups, Type 1 (West African) and Type 2 (East African) (*34*). Recent genetic analysis suggests that *M. africanum* Type 2 (East African) is a phenotypic variant of *M. tuberculosis* and relatively distant from *M. africanum* Type 1 (West African), which is characterized by a deleted RD9, an intact TbD1 region, and specific SNPs in *katG* and *gyrA* genes (*35*,*36*). Our data suggest a more complex phylogeny of *M. africanum* Type 1 (West African). This phylogeny is complicated further by strains with a deleted RD9 and a single copy of MIRU24 originating predominantly from the Indian subcontinent.

The VNTR numbers seen within each spoligo family are shown in Table 3. From these data, lineage-dependent VNTR locus plasticity can be seen. This plasticity ranges from 7/15 loci showing variation within the CAS to 14/15 showing variation in the EuroAm lineage. VNTR loci such as MIRU10 and 16 show variation across all families, whereas MIRU27 shows variation in CAS alone. The distribution of repeat numbers at each locus within each lineage suggests the variation seen has arisen by stepwise mutations of a lineage founder strain. It is likely that the VNTR profiles used to predict spoligotype family at the highest specificity (Table 2) represent this type.

Using country of birth as a surrogate for geographic origin of an infecting strain, we saw strong associations with the lineage/spoligo family of isolates (online appendix Table). The data here confirm published data that Beijing strains were associated with patients originating from Southeast Asia; EAI with patients from Southeast Asia, the Indian subcontinent, and East Africa; CAS with patients from the Indian subcontinent; and EuroAm with a global distribution of patients (*7*,*32*). This global geographic structuring may explain the apparent geographic variation in efficacy of the *M. bovis* BCG vaccine.

It has been long questioned whether there is an association between site and progression of infection and bacterial genotype; some evidence supports this association (*37*,*38*). Our study showed no association between lineage or spoligo family and site of infection.

That the *M. bovis* family was associated with pyrazinamide resistance would be expected because resistance is a defining characteristic for most of the group (although not for *M. bovis* subsp. *caprae)*. Beijing family isolates were associated with multidrug resistance and streptomycin resistance. The association with multidrug resistance has been reported (*8*), but the evidence presented here is particularly compelling, given that all strains used in the analysis were individual types. The value of this approach was demonstrated by analyzing LAM10 isolates, a family to which a highly successful clone of isoniazid-resistant *M. tuberculosis* responsible for >250 cases in northern London (*38*) belongs. Eight isolates were identified in this study. When all isolates belonging to this group were analyzed, LAM10 was strongly associated with isoniazid resistance ($p<0.00001$), but when a single representative of each cluster was used this association disappeared. The Beijing lineage would appear to have a predisposition toward the acquisition of drug resistance rather than the drug-resistant clones being transmitted more frequently. The extent of the geographic regions used in the association study make it unlikely that this predisposition is entirely due to local TB control and treatment practices.

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New Hypothesis for Cause of Epidemic among Native Americans, New England, 1616–1619

John S. Marr and John T. Cathey

In the years before English settlers established the Plymouth colony (1616–1619), most Native Americans living on the southeastern coast of present-day Massachusetts died from a mysterious disease. Classic explanations have included yellow fever, smallpox, and plague. Chickenpox and trichinosis are among more recent proposals. We suggest an additional candidate: leptospirosis complicated by Weil syndrome. Rodent reservoirs from European ships infected indigenous reservoirs and contaminated land and fresh water. Local ecology and high-risk quotidian practices of the native population favored exposure and were not shared by Europeans. Reduction of the population may have been incremental, episodic, and continuous; local customs continuously exposed this population to hyperendemic leptospiral infection over months or years, and only a fraction survived. Previous proposals do not adequately account for signature signs (epistaxis, jaundice) and do not consider customs that may have been instrumental to the near annihilation of Native Americans, which facilitated successful colonization of the Massachusetts Bay area.

Retrospective studies have inherent, sometimes insurmountable, biases, but speculation on past events by historians and anthropologists is commonplace and offers grist for future studies. We offer an alternative hypothesis for the cause of an epidemic among Native Americans in the years immediately before the arrival of the Pilgrims in Massachusetts. During 1616–1619, many persons died of a disease that presumably spared nearby European fishermen and traders (*1*). The more severe manifestations were fever, headache, epistaxis, jaundice, and skin lesions. Speculations as to the cause have included plague, yellow fever, and smallpox (*2–7*), as well as influenza, chickenpox, typhus, typhoid fever, trichinosis, cerebrospinal meningitis, and syndemic infection of hepatitis B virus (HBV) and hepatitis D virus (HDV) (Table 1) (*6–11*). We propose another disease: leptospirosis, accompanied by Weil syndrome. With its more severe manifestations, this syndrome is consistent with available clinical information, the nidality of *Leptospira* organisms, the introduction of rodent reservoirs, and the presence of favorable ecologic niches. Practices of the local population placed it repeatedly in high-risk exposures to epidemic and hyperendemic environments.

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Epidemiology

The limited information available notes the following clinical manifestations of the illness: headache and fever with visible signs of epistaxis and jaundice. Mode of

Table 1. Summary of published interpretations suggesting or discounting possible causes of an epidemic among Native Americans, New England, 1616–1619*

Cause	Suggested	Discounted
Yellow fever	Webster (*2*)	Cook (*1*), Williams (*3*), Carter (*4*), Bratton (*6*)
Plague	Williams (*3*)	Carter (*4*), Hoornbeek (*5*), Bratton (*6*), Crosby (*9*)
Influenza	Carter (*4*)	
Smallpox	Bratton (*6*), Holmes (*7*)	Cook (*1*), Webster (*2*), Williams (*3*), Hoornbeek (*5*)
Chickenpox	Hoornbeek (*5*), Cronon (*10*)	Bratton (*6*)
Typhus	Lescarbot (*11*)	Williams (*3*), Bratton (*6*)
HBV/HDV	Speiss and Speiss (*8*)	
Leptospirosis	This study	

*HBV, hepatitis B virus; HDV, hepatitis D virus.

transmission was not known. Weather and seasonality are unknown, although tree ring data suggest greater than average rainfall in eastern Massachusetts during 1615–1625 (*12*). The duration of the epidemic (or epidemics) reportedly ranged from 3 to 6 years. Estimated death rates (which lack reliable numerator and denominator data) range from one third of the local population to as high as 90% (*1*,*13*). The Patuxet (Plimouth) Native American village was severely depopulated (*14*). Referring to conditions along the Newfoundland and Maine coasts, where some believe the epidemic may have originated, Pierre Biard, a Jesuit missionary, noted: "They [the Indians] are astonished and often complain that since the French mingle and carry on trade with them, they are dying fast, and the population is thinning out" (*15*). In New England, Smith noted "three plagues in three years successively neere two hundred miles along the coast" of southern Massachusetts to Cape Cod and inland for 15 miles (*16*). Bennett suggested a 50–60-mile interior extension, which corresponds to the area of native corn horticulture (*17*).

By 1616, several subtribes of the Wampanoag (Pokanoket) Nation were living between the present-day borders of eastern Rhode Island and southeastern Maine (Figure 1). The Patuxet village was localized to an area in and around Plymouth harbor (Figure 2). Demographers and historians disagree about the total size of the Wampanoag Nation, but Salisbury considers an estimate of 21,000–24,000 as "not unrealistic for this region" (*13*). Gookin also estimated 3,000 men living in Massachusetts before the epidemic (*18*), which when extrapolated for family size is consistent with Salisbury's overall estimate. Salisbury estimated that the size of the Patuxet tribe before the epidemic was 2,000.

No estimates are available of the number of Portuguese, Breton, and Bristol fishermen; Basque whalers; French fur traders; or English codders who had established a presence on the North Atlantic coast since the early sixteenth century (*10*). In 1578, an observer noted 100 Spanish sails, 20–30 Basque whalers, ≈150 French and Breton fishing ships, and 50 English sails along the coast of Newfoundland (*19*). English traders and fishermen had daily contact with indigenous persons but lived on ships or in segregated enclaves on land where salt-dried codfish stations (favored by the English) were built along Massachusetts Bay.

Ecology

Indigenous ecology was cataloged in 1604 when hundreds of coastal plants, trees, and animals (but not "vermine") were described (*20*). Before 1620, there were no peridomiciliary animals except for small dogs and mice (*10*), although other rodents (e.g., squirrels) were common. Precolonization and postcolonization English written accounts do not mention rats, the numbers of which may have been influenced by the presence of cats, but aboard

Figure 1. Native American tribes of southeastern Massachusetts in ≈1620.

ships rats must have been common. An earlier explorer noted "Tant qu'on eut des cuirs on ne s'avisa point de faire la guerre aux rats…" ("As long as there is a cargo of skins, it makes no sense to kill the rats.") (*11*). The black rat (*Rattus rattus*) was common in coastal England at the time (yet to be displaced by the brown rat [*R. norvegicus*] nearly 100 years later) (*21*); the black rat and mice were universal companions on ships and must have established themselves early on the coastal mainland, seeking harborage in and around Native American households. Once established, rats and mice would become chronic carriers of disease agents, contaminating water and soil and infecting other commensal rodents (e.g., the local mouse *Peromyscus leucopus*) and other mammals. Fresh and stored food items such as maize, beans, squash, pumpkin, roots, nuts, berries, meat, fish, and shellfish, were also susceptible to leptospiral contamination.

Previous Explanations

One hundred years ago, Williams collected all known information about the epidemic in an article that included 23 primary references, 22 of which contained eyewitness accounts or reports (*3*). He concluded that the disease may have been bubonic plague and supported his proposal by noting that there were abundant fleas in Indian dwellings, survivors had sores suggestive of buboes, and plague was endemic in London during 1606–1611. Eleven of his 23 primary sources disagreed, as did Carter, who without further elaboration stated that he thought the epidemic was influenza (*4*). Despite allusions to icterus, Williams discounted yellow fever (as did Carter); he also dismissed other febrile illnesses with jaundice, yet he cited Gookin from 1674: "I have discoursed with old Indians, who were then youths, who say that the bodies all over were exceedingly yellow, describing it by a yellow garment they showed me, both

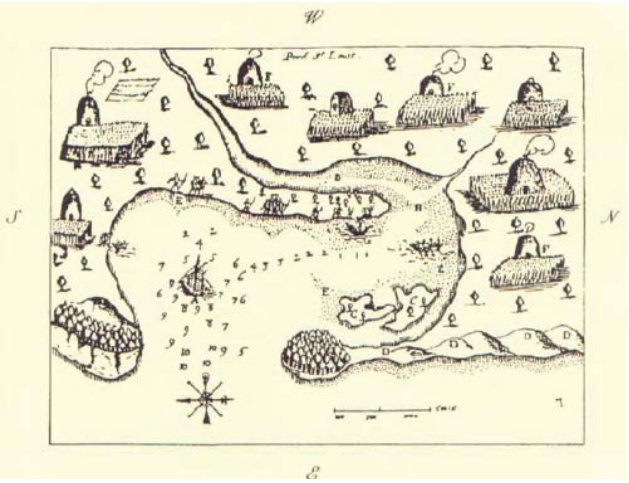

Figure 2. Plymouth, Massachusetts, harbor showing extensive Native American settlement (a sketch by Samuel de Champlain from his voyage of 1606).

before they died and afterwards." Trumbull, another eyewitness, noted that the Indian word for the disease meant "a bad yellowing" (*3*). A recent analysis interpreted it as caused by a confluent form of smallpox (*6*). Clinical and epidemiologic information about classical explanations and some of the more recent suggestions are summarized in Table 2.

Discussion

The causes of most historical epidemics may never be proven. The new science of paleomicrobiology may provide some answers, but the question will remain about whether a person died of a specific disease or with the disease. However, even when proper evidence is limited, this limitation should not dissuade speculation about the causes of ancient afflictions. Our hypothesis is not meant to be a definite answer but a heuristic for others to criticize and explore. Alfred Crosby, one of America's foremost medical historians, coined the term "virgin soil epidemics" to describe immunologically unexposed populations exposed to Old World diseases and cited the 1616–1619 epidemic as an example (*9*). He also proposed that environmental and behavioral factors were equally important (*22*). The Massachusetts epidemic supports this observation, and evidence may indicate that "genetic weakness" was not as important as the intimate and repeated exposure to an infectious agent among the Indians not shared by Europeans.

All previously proposed explanations for the epidemic are consistent with an Old World importation into a susceptible population (except for Webster's, who thought yellow fever was of autochthonous origin). Despite its manifestation and subsequent visitations along coastal America in later years, yellow fever is not a plausible explanation given the routes of the trans-Atlantic slave trade at the time. Transportation of the disease, its vector, and human cargo from Africa to the New World was limited to the Caribbean and Central and South America; little evidence exists that any ships visited the New England coast after disembarking slaves (*23*). Alternative arthropod-borne and other non-arthropod–borne viral hemorrhagic fevers are even less plausible candidates.

Clinical descriptions of other proposed diseases (plague, chickenpox, typhus, typhoid fever, and meningitis) are largely inconsistent with the syndrome described and were dismissed by Bratton. Citing Oliver Wendell Holmes, Sr. (*7*), Bratton concluded that the disease was smallpox, explaining that the confluent form of pustular smallpox might mimic jaundice (*6*). In 1799, Webster had discounted smallpox because "the Indians, who were perfectly acquainted with the disease [smallpox] after the English arrived, always gave a very different account of it..." (*2*). Two diseases not mentioned by Bratton (trichino-

Table 2. Factors related to some of the postulated causes of an epidemic among Native Americans, New England, 1616–1619*

Factor	Yellow fever	Plague	Influenza	Smallpox	Chickenpox	Typhus	HBV/HDV	Leptospirosis
Characteristic signs and symptoms								
Headache/fever	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Jaundice	Yes	No	No	No	No	No	Yes	Yes
Hemorrhages	Yes	Yes	No	Yes	No	Yes	No	Yes
Skin lesions†	Bruises	Buboes	No	Pustules	Rash	Rash	No	Rash
Epidemiologic								
High attack rate	Yes	Yes	Yes	Yes‡	Yes	Yes	Yes	Yes
High death rate	Yes	Yes	No	Yes	No	Yes	Yes	Yes
Endemic in Europe	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Suitable arthropod vector	No	Yes	NA	NA	NA	Yes	NA	NA
Suitable reservoir host	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Native susceptibility	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
European susceptibility	Yes	Yes	Yes	No	No	Yes	Yes	Yes

*HBV, hepatitis B virus; HDV, hepatitis D virus; NA, not applicable.
†Sign mentioned by only 1 person (Thomas Dermer) and possibly referred to another unrelated disease outbreak.
‡Native Americans only.

sis and HBV/HDV infections) are also unlikely. Pigs were absent in the New World, and the finding of a single pig bone in an undated midden makes a most unlikely explanation for the epidemic. Syndemic HBV/HDV infection presupposes aboriginal HBV carriage, HDV importation, and (in the opinion of Speiss and Speiss) an enteric mode of transmission (*8*).

In 1886, Adolf Weil originally described a constellation of signs and symptoms that is now eponymic for Weil syndrome (his first patient experienced nasenbluten [nosebleed] on the second day of illness) (*24*). Inada and Ido identified the causative organism 30 years later (*25*). Subsequent studies have demonstrated that rodents have high rates of leptospiral carriage and shedding (*26*). Severe (icteric) leptospirosis was also known as infectious jaundice, epidemic jaundice, and icto-hemorrhagic fever (*27*). Early outbreaks in the United States were recorded by Neill, including a Union Civil War Surgeon General's report of a large number of "hepatic and haematic disorders" estimated to have affected >71,000 troops during the War (*28*).

In 1965, Heath et al. summarized the history of leptospirosis in the United States, analyzing 483 cases reported during 1949–1961 (*29*,*30*). Twenty-five percent were caused by *L*. serovar Icterohemorrhagiae. Today, *L*. Icterohemorrhagiae and other serovars (Canicola, Autumnalis, Hebdomidis, Australis, and Pomona) are endemic in the United States, and isolated instances within the United States continue to be reported (*31*). More recent reports from the Centers for Disease Control and Prevention (*32*,*33*) and ProMED mail (*34*) demonstrate that leptospirosis is a worldwide, reemerging infection with identifiable risk factors, including immersion in fresh water, exposure to contaminated soil, and antecedent heavy rains (*35*,*36*). Unlike hookworm disease, another Old World soil-borne disease that established itself in the more hospitable American South, leptospirosis is a more cosmopolitan fellow traveler and is still recognized as a zoonosis in New England.

Contemporary medical texts conflate signs, symptoms, and death rates of mild leptospiral infection with Weil syndrome, relying on more recent citations in which the nature of exposure, duration, and responsible *Leptospira* spp. are often not known. Interventional measures (removal from known sources, prompt diagnosis and treatment, and early prevention and control measures) may have decreased overall case-fatality rates and limited the extent of the outbreaks. Nosebleed is rarely mentioned in the recent literature, but "hemorrhages, starting with epistaxis" are noted in a 1944 text on tropical diseases, which also cites high death rates (32% in Europe and 48% in Japan) (*27*). These surprisingly high death rates in early Japanese reports were attributed to repeated intimate exposure to contaminated water by barefooted mine workers and rice farmers.

Unlike the European experience, epidemics in Japan were rare, and endemic exposures were more common (*27*). A recent population-based seroepidemiologic study found leptospiral seropositivity rates of 28% in an annually flooded area of the Amazon basin (*37*). *Leptospira* spp. were found to cause seasonal outbreaks of a mysterious disease (tentatively named Andaman hemorrhagic fever) during periods of rice paddy sowing and harvesting in the late 1980s on the Andaman Islands in the Indian Ocean (*38*). Subsequent studies found that leptospiral seroposivity was as high as 62.5% (among agricultural workers) in the Andaman Islands and that the case-fatality rate was 42.9% among hospitalized patients with severe leptospirosis and pulmonary symptoms.

Endemicity and subsequent high case-fatality rates, similar to those reported from Japan, are consistent with a leptospiral etiology for the 1616–1619 epidemic. The Patuxets may not have associated sickness with their environment or traditional ways of living and may have attributed their affliction to many causes, but not to countless exposures and reexposures to the agent. Sporadic, focal mini-epidemics may have played out and coalesced into what was construed as a single "plague" by outside observers. Except for more severe cases of liver failure, the most common cause of death for leptospirosis (renal or respiratory insufficiency) would have not been recognized. The Indian lifestyle, which included constant exposure to rodents and their excreta on land and in water, exposed them to the leptospiral life cycle (Figure 3) (*39*,*40*). Bare feet were common in and around houses. Although a rare portal of entry, mucosal exposure may have occurred from ingestion of corn buried in the ground in rodent-accessible baskets and from rodent-contaminated foods in wigwams (weetas). Dermal abrasions offered cutaneous portals of entry. Attendance of the ill and burial of the dead (including those who died from Weil syndrome) would have attracted others who shared local food, water, and camp grounds. It was common practice for entire families to enter sweat lodges followed by immediate immersion in cooling streams and ponds; sweat lodges were considered vivifiers and cure-alls for illnesses, a practice that may have reexposed the already ill to contaminated water. Once the spirochete established its presence in numerous foci, it survived for months in water, mud, and moist soil and caused infection in additional mammalian reservoirs. A reduction in the populace may have been incremental, episodic, and continuous; daily needs and customs may have exposed the Indians to leptospirosis over many months or years, with only a small fraction of the population eventually surviving. Suggestions that the disease persisted among the Indians after 1619 (perhaps through 1630) support the premise of endemic nidality and selective Indian vulnerability. The fate of nearby European cod fishermen is unknown, but they did not share

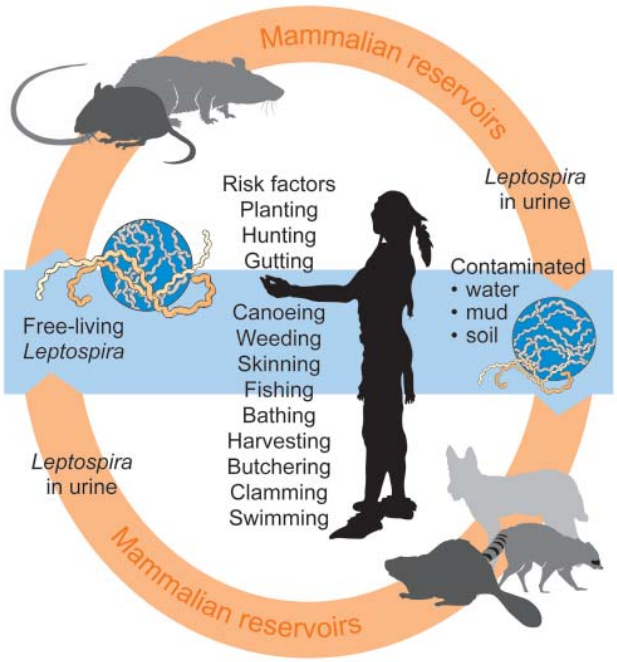

Figure 3. Leptospiral life cycle.

most of the Indians' risk factors. Boots would have limited transmission from fresh water exposures, bathing was not a common practice, and work in a saline environment may have curtailed transmission. An occasional case of febrile illness on board ship would have been attributed to many other causes. Disease and death may have occurred among the fishermen but are not recorded.

The exact duration and extent of the epidemic(s) will never be known, but our suggestion offers an alternative explanation. Persistent leptospiral exposures resulted in more severe cases of Weil syndrome and jaundice, a sign that would have been reported by observers; the cause of death from other (anicteric) leptospiral infection would not have been recognized. Our proposal is consistent with the historical clinical descriptions, estimated death rates, importation and distribution of its reservoir host, inoculation of the agent in multiple suitable nidalities, spread to other mammalian reservoirs, hyperendemicity, ecologic factors favoring repeated exposure and transmission, and known high-risk activities of the indigenous population.

The name Squanto has entered American history and folklore as the one of the last of the Patuxets who assisted the Pilgrims in 1620. He was one of the few survivors of an epidemic that was crucial to the success of the Plymouth and Massachusetts Bay colonies because remaining Indians had little capacity to resist the new settlers. Two years later, after having fever and a nosebleed, Squanto died of what was then referred to as "the Indean disease."

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etymologia

Cryptococcus gattii

[krip″ to-kok′əs ga-te-i]

This yeast genus takes its name from the Greek *kryptos*, hidden, and *kokkos*, berry. The pathogen has been recently recognized as a distinct species that causes infection (with cutaneous, pulmonary, and neurologic manifestations) in both humans and animals. The species was named for Italian mycologist Franco Gatti who, with Roger Eeckels, described an atypical strain of *C. neoformans* in the cerebrospinal fluid of a Congolese Bantu boy with cryptococcosis in 1970.

Sources: Vanbruseghem R, Takashio M. An atypical strain of *Cryptococcus neoformans* (San Felice) Vuillemin 1894. Part II. *Cryptococcus neoformans* var *gattii* var. nov. Am Soc Belge Med Trop. 1970;50:695–702; Springer DJ, Chaturvedi V. Projecting global occurrence *Cryptococcus gattii*. Emerg Infect Dis. 2010;16:14–20; Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders Elsevier; 2007.

Clonal Distribution of Invasive Pneumococci, Czech Republic, 1996–2003

Helena Žemličková, Pavla Urbášková, Vladislav Jakubů, Jitka Motlová, Martin Musílek, and Bohumír Procházka

We conducted surveillance on invasive pneumococci isolated from adults in the Czech Republic during 1996–2003. The 7 most prevalent serotypes were characterized. Coverage with the 7-valent pneumococcal conjugate vaccine was low. Our observations confirm that detection methods may have modified the expected effect of this vaccine.

Streptococcus pneumoniae is a leading cause of illness and death in children and adults (*1*,*2*). The incidence of invasive pneumococcal disease (IPD) has been reduced by a 7-valent pneumococcal conjugate vaccine (PCV7) (*3*). Although certain serotypes of *S. pneumoniae* are recognized among children, adults carry a wider range of serotypes (*4*). Several studies have reported cases of IPD in adults caused by common childhood serotypes (*5*,*6*). The causes of such cases are unclear, but use of antimicrobial drugs (*6*), progressive population aging (*5*), and modifications to blood culture protocols have been suggested (*7*). PCV7 efficacy in children has raised the question of whether to use PCV7 in elderly persons (*5*).

In the Czech Republic, patients in follow-up care departments (i.e., <1% of the adult population), have been vaccinated with a 23-valent polysaccharide vaccine since 2001. PCV7 showed a reported 66% serotype coverage in children (*8*) and has been included in the routine childhood immunization program since January 2009.

Clones associated with IPD have been characterized by genotyping. Use of PCV7 exerts selective pressure against vaccine serotypes. Clonality data obtained before vaccine use will make it possible to demonstrate future changes in the genetic structure of pneumococci. Therefore, the purpose of our study was to identify IPD serotype distribution and clonality in adults in the Czech Republic before the use of PCV7.

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The Study

Invasive pneumococcal isolates from adults ≥18 years of age were obtained from blood (n = 460) and cerebrospinal fluid (CSF) (n = 229) during 1996–2003 by 54 laboratories serving ≈85% of the population in the Czech Republic. Isolates were serotyped by using the Quellung reaction (*9*). Antimicrobial drug susceptibility and MICs were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute recommendations (*10*). Genotyping was performed by using pulsed-field gel electrophoresis (PFGE) (*11*) and multilocus sequence typing (MLST) (*12*). MLST results were identical between isolates of the same serotype within a PFGE subtype (*13*). Cluster analyses were based on allelic data that used the eBURST algorithm (*14*). Numerical trend was evaluated by regression analysis of the logarithm of the number of isolates by using SPSS for Windows version 14 (SPSS, Inc., Chicago, IL, USA). The χ^2 test for trend (Epi Info version 3.4; Centers for Disease Control and Prevention, Atlanta, GA, USA) was used to evaluate variations in proportions of different serotypes; p values <0.05 were considered significant.

During 1996–2003, a total of 446 (64.7%) isolates were obtained from adults 18–64 years of age and 243 (35.3%) isolates were obtained from adults ≥65 years of age. A progressive increase in recovery of isolates from blood was observed from 44 during 1996–1997 to 199 during 2002–2003 (p = 0.009) (Figure 1). Serotyping identified 48 serotypes; 10 of these serotypes included ≥30 isolates. Half of all strains belonged to serotypes 3 (12.7%), 4 (8.6%), 8 (7%), 1 (5.8%), 19F (5.5%), 14 (5.5%), and 9V (5.2%) (Figure 2). Serotype coverage rates by PCV7, PCV10, PCV13, and 23-valent polysaccharide vaccine were 32.5%, 44.2%, 62.3%, and 82.1%, respectively, among adults 18–64 years of age and 35.4%, 42.8%, 61.3%, and 85.6%, respectively, among adults ≥65 years of age.

Serotype distribution did not vary significantly with age group and type of isolate, except for serotype 1, which was more common (p = 0.037) among younger adults (18–64 years) and more prevalent in blood (p = 0.004). With the exception of 1998–1999, serotype 3 was most common identified. This serotype, which composed≈11% of all isolates during 1996–2001, increased to 15% during 2002–2003 (p = 0.289). A change was also observed in the proportions of serotypes 1 and 4 (Figure 1). Serotype 1 increased from 6% during 1996–1997 to 17% during 1998–1999 (p = 0.027) and then decreased to 3% during 2002–2003 (p<0.001). Serotype 4 increased from 2% during 1996–1997 to 13% during 2002–2003 (p<0.001) (Figure 1).

Penicillin-resistant pneumococci were uncommon (range 1%–5%), and no isolate showed high resistance to penicillin (MIC >1 mg/L). Resistance to chloramphenicol decreased from 5% during 1996–1997 to 2% during 2002–

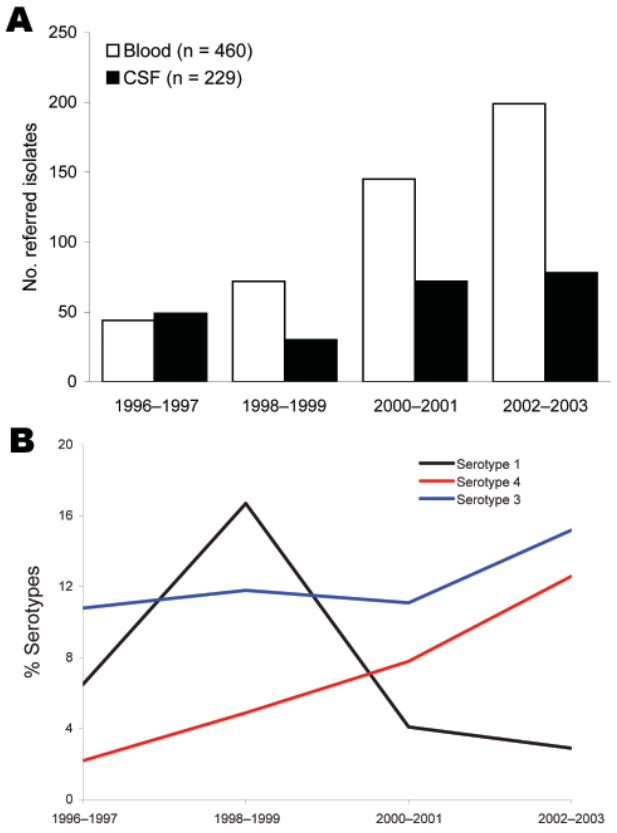

Figure 1. A) Invasive pneumococcal isolates from blood and cerebrospinal fluid (CSF) and B) frequency of *Streptococcus pneumoniae* serotypes 1, 3, and 4 among adults, Czech Republic, 1996–2003.

2003 (p = 0.114). Tetracycline resistance also decreased from 8% during 1996–1997 to 6% during 2002–2003 (p = 0.732). Few (≤9) isolates were resistant to erythromycin. Antimicrobial drug resistance was associated with certain serotypes. Eighteen of 23 penicillin-resistant pneumococci isolates belonged to serotype 9V. Resistance to chloramphenicol, usually observed with resistance to tetracycline, was seen most often in serotype 19F isolates.

Of 347 isolates of the 7 most common serotypes, 335 were characterized by PFGE and 130 PFGE subtypes were analyzed by MLST (online Appendix Table, www.cdc.gov/EID/content/16/2/287-appT.htm). Forty-six allelic profiles were identified, 18 of which carried new alleles (n = 11) or novel combinations of known alleles (n = 7). Of 10 new alleles, 5 belonged to serotype 4. The *gdh128* allele was identified in serotype 4 and 9V isolates. Until recently, only *gdh128* was found among isolates from other countries. At least 60% of isolates of serotypes 1, 3, 9V, 14, and 19F have been identified within clones in the Pneumococcal Molecular Epidemiology Network. In contrast, only 25% of serotype 4 and 8 isolates were identified in this network, and these serotypes consisted of larger clusters unrelated to global clones.

Conclusions

When we compared our results with those of a study from the United States (*5*), where 51% of IPD was among elderly persons before introduction of PCV7 and caused by pediatric serotypes (6B, 9V, 14, 19F, and 23F) (*5*), our data showed a low frequency (27.2%) of pediatric serotypes in older adults. The low frequency of penicillin-nonsusceptible pneumococci isolates of pediatric serotypes explains the observed predominance of drug-susceptible serotypes. Local differences in blood culture protocols could also bias the serotype distribution (*7*). Although the number of blood cultures performed in the Czech Republic is increasing, lower numbers of invasive pneumococci have been isolated than in other countries in Europe (www.rivm.nl/earss).

The observed predominance of serotype 3, previously reported as less invasive but associated with a high death rate (*15*), could be explained by sample collection primarily from the most severe IPD cases. In 1998, an increase in serotype 1 invasive disease was reported in Sweden. In our study, serotype 1 was the most common during the

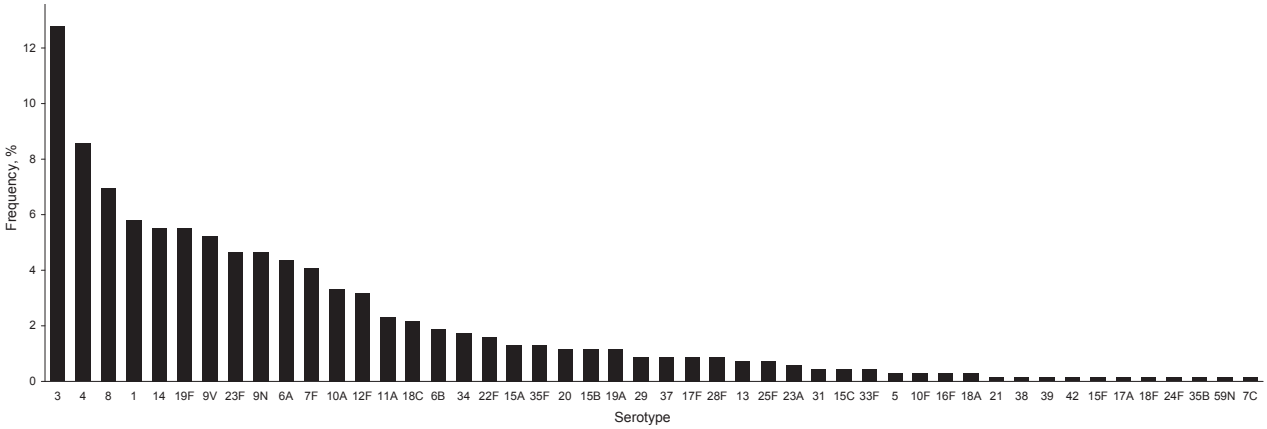

Figure 2. Distribution of pneumococcal invasive serotypes among 689 adults, Czech Republic, 1996–2003.

same period. Subsequently, the frequency of this serotype decreased but the frequency of bacteremia increased. The gradual increase in bacteremia and increased frequency of serotype 4, previously found to be moderately invasive (*7*), could be ascribed to the increase in blood cultures that facilitated detection of moderate disease caused by serotype 4 rather than dissemination of a specific invasive clone because the isolates showed high genotypic diversity. In our collection of invasive strains, the frequency of antimicrobial drug–resistant global clones, all previously found in the Czech Republic (*13*), varied between serotypes.

Our results support the need for IPD surveillance because differences in detection methods might modify the effect of the vaccine. Continued surveillance will be helpful in detecting any shift in the distribution of invasive serotypes and clones circulating in the Czech Republic and in monitoring the effect of PCV7 on serotype frequencies and the genetic structure of pneumococci.

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White-Nose Syndrome Fungus (*Geomyces destructans*) in Bat, France

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White-nose syndrome is caused by the fungus *Geomyces destructans* and is responsible for the deaths of >1,000,000 bats since 2006. This disease and fungus had been restricted to the northeastern United States. We detected this fungus in a bat in France and assessed the implications of this finding.

Biologists are struggling to understand a recent emerging infectious disease, white-nose syndrome (WNS) (*1*), which potentially threatens >20% of all mammalian diversity (bats) (*2*). WNS is a deadly epidemic that has swept through the northeastern United States over the past 3 years and caused the death of >1,000,000 bats, with decreases of ≈100% in some populations (*3*).

This disease is associated with hibernating, cave-roosting bats. A visually conspicuous white fungus grows on the face, ears, or wings of stricken bats; infiltration of the hyphae into membranes and tissues leads to severe damage (*4*). Bats that exhibit WNS have little or no fat reserves, which are essential for their survival throughout and after hibernation (*5*). The fungus associated with WNS is a newly described, psychrophilic (cold-loving) species (*Geomyces destructans*) (*6*). It is closely related to *G. pannorum*, which causes skin infections in humans (*7*).

Although it is not known whether the fungus is primarily responsible for deaths of bats or is a secondary infection, it is directly associated with deaths of bats (*5*). Bacteriologic, virologic, parasitologic, and postmortem evaluations for the cause of death did not identify any other agents, which reinforces the suspicion that this fungus is the causative agent (*4*,*5*). To date, WNS has been found only in the northeastern United States. However, researchers have suggested its presence in Europe. We investigated whether *G. destructans* is present in bats in Europe and assessed the implications of its presence.

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The Study

During intensive monitoring of bat hibernation in France, 1 bat (*Myotis myotis*) found on March 12, 2009, near Périgueux (45°8′N, 0°44′E), showed a powdery, white fungal growth on its nose (Figure 1, panel A), which is characteristic of WNS. Sterile dry cotton swabs were used to collect fungus material from the nose of the bat. The bat was then weighed, measured, and released. Swabs were moistened with 50 μL of sterile water and streaked onto plates containing potato dextrose agar supplemented with 0.1% mycologic peptone. Plates (9 cm in diameter) were sealed with parafilm and incubated inverted at 10°C. A dense fungus growth developed within 14 days (Figure 1, panel B).

Cultures were established by transferring inoculum to other mycologic media, including malt extract agar and Sabouraud agar. Colonies on malt extract agar were initially white but after spore production and aging they quickly darkened from the center to a dull gray, often showing a faint green hue. Spores were hyaline, irregularly curved, broadly crescent-shaped (typically 6–8 μm long and 3–4 μm wide), and narrowed at each end, one of which was broadly truncate, often showing an annular frill (Figure 1, panel C). Fungal cultures have been deposited in the culture collection of the Industrial Microbiology Department of University College Dublin (Reference IMD Z2053).

Microscopic examination of the original swab samples showed numerous spores with the above-mentioned features. The psychrophilic nature of the fungus and its species-specific morphologic features (online Technical Appendix, www.cdc.gov/EID/content/16/2/290-Techapp.pdf) led to the conclusion that this fungus was *G. destructans*, which was recently isolated from WNS-positive bats in the northeastern United States (*6*).

Two molecular markers were sequenced from 6 randomly chosen fungus cultures to confirm species identity. DNA was extracted by using a Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with slight modifications (after step 3, we added an incubation time of 10 min at 70°C). The internal transcribed spacer (ITS) regions (ITS1, 5.8S, and ITS2) and the small subunit (SSU) of the rRNA gene were amplified separately.

PCRs were performed in 25-μL volumes containing 1 μL of DNA (10–75 ng/μL), 1.5 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, 0.2 mmol/L dNTP, 1× PCR buffer, and 1 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carslbad, CA, USA). Identical PCR cycling conditions were used for both fragments: an initial step at 95°C for 15 s; 10 cycles at 95°C for 30 s, 60°C (reduction of 2°C

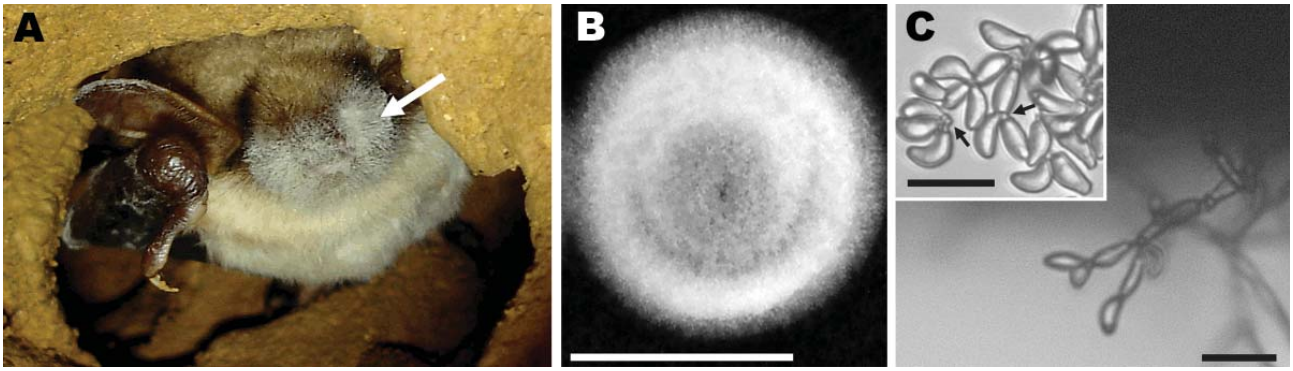

Figure 1. A) *Myotis myotis* bat found in a cave on March 12, 2009, in France, showing white fungal growth on its nose (arrow). B) Fungus colony on malt extract medium after incubation for 3 weeks at 10°C. Scale bar = 1 cm. C) Clusters of unstained spores of *Geomyces destructans*. Spores in the inset were stained with lactophenol cotton blue, which shows the truncate spore base (arrows) and surface granulation. Scale bars = 10 μm.

every 2 cycles) for 1 min and 45 s, and 72°C for 1 min; 30 cycles at 95°C for 30 s, 50°C for 1 min and 45 s, and 72°C for 1 min; and a final step at 72°C for 10 min. PCR products were purified and sequenced in both directions by using primers listed in the Table. Complementary sequences were assembled and edited for accuracy by using CodonCode Aligner version 3.0.3 (www.codoncode.com/aligner/download.htm).

The ITS and SSU sequences from the 6 WNS fungus cultures were identical. They were deposited in GenBank as single sequences: ITS (GQ489024) and SSU (GQ489025). Sequences obtained for the 2 genetic markers showed a 100% sequence identity with the described *G. destructans* fungus (Figure 2, panels A, B). Thus, morphologic and genetic data support the presence of *G. destructans* infection in a bat in France.

Conclusions

Our results show that the WNS fungus was present in a bat in France and has implications for WNS research, bat conservation, and emerging infectious disease control. We suggest 3 possible scenarios for our findings. The first scenario is that the fungus has only recently arrived in Europe and all bats in Europe are now at risk for infection. Thus, conservation steps must be taken to minimize the spread of this disease, especially because this disease is specific for hibernating bats. After the hibernation period, *M. myotis* bats may migrate up to 436 km to reach their summer roosts (*10*), a behavior that could quickly increase the chance of fungus transmission. A second scenario is that the fungus has been present in Europe for a long time. Because mass deaths have not been observed in bats in Europe, these bats may be immune to WNS. Therefore, identification of mechanisms of this immunity will advance understanding of this disease and fungus resistance in mammals. The third scenario is that the *G. destructans* fungus is not the primary cause of death but acts as an opportunistic pathogen in bats already immunocompromised by other pathogens such as viruses or bacteria (*1*). Comparison of pathogens in bats in Europe and the United States infected with *G. destructans* should identify the primary causative agent.

The bat in our study showing fungal growth was not underweight (Figure 2, panel C), as is typical of bats in the United States with WNS (*4*). This finding favors the second or third scenarios. Also, a 6-year (2004–2009) annual monitoring program of wintering bat populations at the site and 5 sites within a 2-km radius did not show any cases of WNS or deaths and showed stable bat populations. The 3 scenarios indicate that studying *G. destructans* in bats in Europe and the United States is necessary to understand and control this disease.

Another fungus, *Batrachochytrium dendrobatidis*, is the etiologic agent responsible for chytridiomycosis (*11*), which currently threatens >50% of all amphibian species and is primarily responsible for the global decrease and extinction of >200 amphibian species in the past decade (*12*). Because bats account for >20% of mammalian diversity (*2*) and play major roles in ecosystem functions, we need to understand, monitor, and control the progression of WNS. Otherwise, we may be faced with similar unprec-

Table. Primers used for PCR amplification and sequencing of fungus in bats, France*

Gene	Primer sequence (5′ → 3′)	PCR
ITS	TCCTCCGCTTATTGATATGC	Forward
	GGAAGTAAAAGTCGTAACAAGG	Reverse
SSU rRNA	CTGGTTGATTCTGCCAGT	Forward
	AAACCTTGTTACGACTTTTA	Reverse
	CCGGAGAAGGAGCCTGAGAAAC	†
	AACTTAAAGGAATTGACGGAAG	†
	CTCATTCCAATTACAAGACC	†
	GAGTTTCCCCGTGTTGAGTC	†

*ITS, internal transcribed spacer; SSU, small subunit.
†Used for sequencing only.

edented and irreversible losses of mammalian biodiversity and entire ecosystems. Because bats control insect populations throughout the world (*13*–*15*), a large decrease in bat populations would result in insect proliferations that would damage agricultural crops and spread many insect-borne diseases.

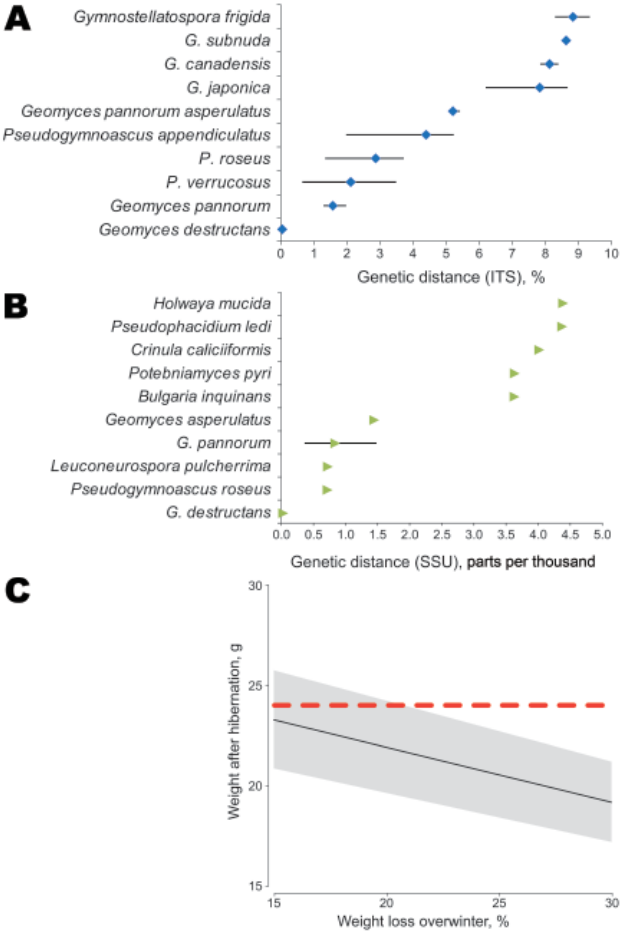

Figure 2. Genetic distance between fungus A) internal transcribed spacer (ITS) (474 nt) and B) small subunit (SSU) rRNA (1,865 nt) gene sequences and other closely related fungus species present in GenBank. Results are based on pairwise sequence comparisons with gaps and missing data removed. Error bars in panels A and B indicate mean ± SD. C) Estimation of weight of *Myotis myotis* bats after hibernation as a function of the range of percentage of weight loss reported. Posthibernation of a bat's weight was estimated from prehibernation measured weights (n = 155 bats) minus winter fat loss. A strong positive relationship exists between body mass and fat mass during prehibernation (*8*). Fat reserves between 15% and 30% of body mass at the onset of hibernation have been reported to be necessary for *Myotis* species to survive winter (*9*). The posthibernation weight (W_{post}) was thus estimated as $W_{post} = W_{pre} – (W_{pre} \times W_{loss}/100)$, where W_{pre} is prehibernation weight and W_{loss} is percentage of body mass lost during hibernation. Mean ± SD prehibernation weight of 155 bats captured in France during August–October 2009 (27.42 ± 2.87 g) was used for the estimate. Black line represents the mean, gray area represents the mean ± SD, and red dashed line represents the 24-g weight of the bat caught in France with white-nose syndrome posthibernation. The bat was in good condition (24 g) because it weighed more than the expected average for a posthibernating bat despite having *Geomyces destructans* growth on its snout.

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Increasing Incidence of Nontuberculous Mycobacteria, Taiwan, 2000–2008

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To assess the species distribution and epidemiologic trends of nontuberculous mycobacteria, we examined isolates from patients in Taiwan. During 2000–2008, the proportion increased significantly from 32.3% to 49.8%. Associated disease incidence increased from 2.7 to 10.2 cases per 100,000 patients. *Mycobacterium avium* complex and *M. abscessus* were most frequently isolated.

In Taiwan, the incidence of tuberculosis (TB) remains high despite advances in the antimycobacterial therapy and implementation of well-known TB control measures (*1*). Therefore, patients with acid-fast bacilli (AFB)–positive specimens, especially respiratory samples, are generally presumed to be infected with *Mycobacterium tuberculosis* and are treated with antituberculosis agents and placed in isolation rooms. Increased isolation of nontuberculous mycobacteria (NTM) causing mycobacterial diseases (*2*,*3*) implies that more patients with AFB-positive samples have received inappropriate or unnecessary empirical antituberculous treatment.

To help clinicians determine whether to initiate antituberculous treatment, mycobacterial laboratories should provide percentages of NTM isolates among specimens with positive AFB. However, the number and species of NTM from clinical specimens are increasing in the clinical laboratory, and the distribution of NTM species varies by geographic area (*4*). We thus sought to assess the species distribution of NTM isolates from various clinical specimens and to elucidate the epidemiologic trends of NTM isolates and diseases over a 9-year period in Taiwan.

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The Study

To assess prevalence of NTM isolates, we used the database of the National Taiwan University Hospital mycobacterial laboratory and prepared clinical specimens for mycobacteria cultures according to recommended guidelines (*5*). Before 2001, specimens were spread onto Lowenstein-Jensen slants and Middlebrook 7H11 medium (BBL; Becton, Dickinson and Company Diagnostic Instrument Systems, Sparks, MD, USA); since late 2001, they have been spread onto Lowenstein-Jensen slants and tested by the fluorometric BACTEC system (BACTEC *Mycobacterium* Growth Indicator Tube 960 system; Becton, Dickinson and Company). NTM were identified to the species level by using conventional biochemical methods. Some unidentified NTM species were further confirmed by sequencing of their 16S rRNA gene (1,464 bp) by using 2 primers (8FPL and 1492) as previously described (*6*,*7*). Diseases caused by NTM were defined according to previous description (*4*). Annual incidence and isolation ratio of TB and NTM over time were evaluated by the Cochran-Armitage test for trend. We considered $p<0.05$ to be significant. To define NTM disease incidence and prevalence, for patients who had isolates over successive years during the study period we included only the year in which they first appeared in the laboratory database.

From January 2000 through December 2008, the laboratory received 283,394 clinical samples for mycobacterial culture. From a total of 23,499 (8.3%) specimens with positive mycobacterial culture results, *M. tuberculosis* were isolated from 14,295 (5.0%) specimens from 3,695 patients and NTM were isolated from 9,204 (3.2%) specimens from 4,786 patients. The trends of decreasing *M. tuberculosis* isolation and increasing NTM isolation were significant ($p<0.05$ for each) (Figure, panel A).

Among the 9,204 NTM isolates, *M. avium* complex (MAC, 30.0%) were the most frequently isolated organisms, followed by *M. abscessus* (17.5%), *M. fortuitum* complex (13.0%), *M. chelonae* complex (9.6%), *M. kansasii* (5.6%), and *M. gordonae* (5.5%) (Table). Among NTM isolates from the 4,786 patients, prevalent species were MAC (31.7%), *M. fortuitum* complex (18.2%), *M. abscessus* (17.2%), *M. gordonae* (11.6%), *M. chelonae* complex (8.2%), and *M. kansasii* (6.0%) (Table). In addition, some rare isolates such as *M. celatum* (n = 3), *M conceptionene* (n = 3), *M. neoaurum* (n = 2), *M. arupense* (n = 1), *M. mageritense* (n = 1), *M. asiaticum* (n = 1), and *M. immunogenum* (n = 1) were identified.

Annual incidences are shown in the Figure, panels B and C. During the study period, the proportions of NTM and rapidly growing mycobacteria among all mycobacteria isolated increased significantly from 32.3% to 49.8% ($p<0.05$) and from 3.7% to 23.4% ($p<0.05$), respectively. Incidence of diseases caused by NTM also increased

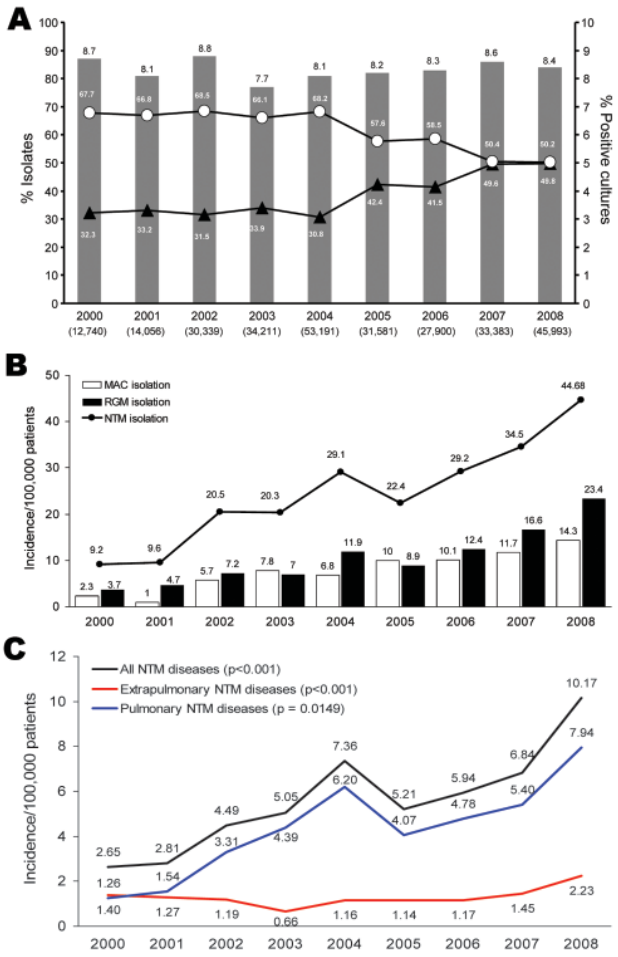

Figure. Incidence data for clinical samples submitted for mycobacterial culture, National Taiwan University Hospital, Taipei, Taiwan, January 2000–December 2008. A) Annual number and rate of isolates of nontuberculous mycobacteria (NTM) (triangles) and *Mycobacterium tuberculosis* (circles). B) Annual incidence of isolates of NTM, *M. avium* complex (MAC), and rapidly growing mycobacteria (RGM). C) Annual incidence of NTM disease, pulmonary disease, and extrapulmonary disease.

(p<0.0001) from 2.7 to 10.2 per 100,000 inpatients and outpatients. The incidences of pulmonary and extrapulmonary NTM infection significantly increased from 1.26 to 7.94 per 100,000 inpatients (p<0.0001) and from 1.4 to 2.23 per 100,000 outpatients (p = 0.0194), and the increase of all NTM diseases was predominately in pulmonary diseases. During the 9 years, 1,105 patients had NTM diseases; most commonly pulmonary disease (n = 894, 76.8%), followed by soft tissue infection (n = 122, 11.4%), disseminated infection (n = 79, 7.1%), and peritonitis (n = 19, 1.7%). MAC was the most common cause of pulmonary infection (n = 342, 40.3%) and disseminated infection (n = 56, 70.1%); *M. abscessus* was the most common cause of skin and soft tissue infection (n = 46, 37.7%).

Conclusions

During the 9-year period, NTM accounted for 39.2% of positive mycobacterial cultures and increased significantly. In concordance with the increased incidence of NTM isolations, incidence of NTM diseases also increased significantly. The increase of NTM isolations since 2002 might have resulted from use of the BACTEC system. This apparent increase in NTM disease could also be attributed to increasing vigilance and awareness of these bacteria as human pathogens, improved methods of detection, or more immunocompromised hosts (e.g., as a result of tumor necrosis factor inhibitors, human interleukin 1 receptor antagonists, and anti-CD20 antibodies) (*1*,*8*).

Prevalence of mycobacteria species responsible for different diseases varies markedly by geographic region. In the United States and Japan, MAC and *M. kansasii* are the most common species (*4*), whereas in England and Scotland, *M. kansasii* and *M. malmoens*e, respectively, are the most common (*9*). Our study showed that MAC was the most common NTM species in Taiwan, followed by *M. abscessus*. The most common organism in localized pulmonary infection and disseminated infection was MAC, and *M. abscessus* predominated in skin and soft tissue infection and lymphadenitis, consistent with findings of a previous study in Taiwan (*2*). Thus, *M. abscessus* deserves as much attention as MAC, especially for extrapulmonary NTM disease, in Taiwan.

Identification of clinical isolates beyond the genus level is crucial because NTM species differ in the clinical spectrum of the diseases they cause and in their susceptibility to antimicrobial drugs. Previous studies have demonstrated that the rare strains identified in this study are pathogenic and cause human infections, e.g., 1 case of catheter-related bloodstream infection caused by *M. neoaurum*, 1 case of pulmonary infection caused by *M. celatum*, and 2 cases of soft tissue infection caused by *M. conceptionene* and *M. arupense* (*10*–*13*). In addition, in this study *M. mageritense* and *M. immunogenum* were the causative agents for pulmonary infection in an adult and submandibular abscess in a child from Taiwan, respectively.

One study limitation was lack of a quantifiable denominator, which is critical for understanding the epidemiology of an illness. True population-based inferences about NTM epidemiology are usually impossible to conclude from a study of only hospital inpatients and outpatients. Although the hospital has a reference mycobacteriology laboratory and is a major referral center in Taiwan, how the study sample may have affected the results or the approximate size of its catchment of patients remains unknown. During the study period, an increasing percentage of patients (≈20% in 2008) was referred from other hospitals in different parts of Taiwan. However, because this study was conducted in a tertiary-care center in northern

Table. Frequently isolated nontuberculous mycobacteria, Taiwan

Bacterial species	Year, no. isolates/no. patients (% isolates/% patients)								
	2000	2001	2002	2003	2004	2005	2006	2007	2008
Mycobacterium avium complex	102/50 (28.5/25.3)	38/23 (10.1/10.1)	244/134 (28.7/27.6)	328/154 (36.7/38.3)	340/158 (25.6/23.2)	449/246 (40.1/44.7)	314/199 (32.7/34.6)	375/242 (26.3/33.8)	571/309 (29.7/32.1)
Mycobacterium abscessus	60/36 (16.8/18.2)	94/51 (24.9/23.3)	123/69 (14.6/14.2)	139/63 (15.6/15.6)	163/85 (12.3/12.5)	181/91 (16.5/16.5)	199/98 (20.7/17.0)	250/140 (16.9/19.6)	400/191 (20.8/19.8)
Total	358/198	378/219	841/485	893/402	1,327/679	1,098/550	961/575	1,424/716	1,924/962

Taiwan, these findings might not reflect the overall situation in Taiwan.

As prevalence and incidence of NTM increases, clinicians in Taiwan should consider NTM as a possible cause of TB-like disease. Accurate species identification is imperative before proper treatment can be determined for diseases caused by the diversity of NTM species. Further studies of clinical isolates are also needed to understand the spectrum of disease caused by these rare pathogens.

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Dr Lai is an attending physician at the Cardinal-Tien Hospital. His primary research interest is clinical infectious diseases, especially tuberculosis, nocardiosis, and diseases caused by nontuberculous mycobacteria.

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Bordetella pertussis Clones Identified by Multilocus Variable-Number Tandem-Repeat Analysis

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Multilocus variable-number tandem-repeat analysis (MLVA) of 316 *Bordetella pertussis* isolates collected over 40 years from Australia and 3 other continents identified 66 MLVA types (MTs), including 6 predominant MTs. Typing of genes encoding acellular vaccine antigens showed changes that may be vaccine driven in 2 MTs prevalent in Australia.

Despite longstanding vaccination programs, pertussis remains endemic to many industrialized countries, including Australia, Canada, Italy, Japan, the Netherlands, Switzerland, and the United States, all of which have reported recent increases in incidence (*1*). Although pertussis is classically a disease of infants and children, this increase has been mainly among adults and adolescents (*2*,*3*). Factors contributing to pertussis resurgence remain unclear, but possible causes are waning immunity, suboptimal vaccine coverage, improved surveillance and diagnosis, the switch from whole cell vaccine (WCV) to acellular vaccine (ACV), and adaptation of circulating *Bordetella pertussis* strains (*4*–*9*). To determine the global epidemiology of pertussis, we analyzed an international collection of *B. pertussis* isolates collected mainly over the past 40 years.

The Study

We used 8 variable-number tandem-repeats, including 6 from Schouls et al. (*10*), to develop a multiplex PCR multilocus variable-number tandem-repeats analysis (MLVA) assay (Table 1; online Technical Appendix, www.cdc.gov/EID/content/16/2/297-Techapp.pdf) and used it to characterize 316 *B. pertussis* isolates from 12 countries on 4 continents, including 208 isolates from Australia and 87 isolates representative of common pulsed field gel electrophoresis types from Canada, Japan, Finland, and the United States (complete list available from authors). The Simpson index of diversity (*D*) ranged from 0.02 to 0.73 per locus with a combined *D* of 0.911 (Table 2). The isolates were resolved into 66 MLVA types (MTs) (complete list available from authors). Thirty-five MTs were represented by single isolates, including 15 of 208 isolates from Australia and 10 of 49 isolates from Japan. Thirty-seven MTs were previously found in Europe (*10*,*11*) and 27 were novel. Fourteen MTs were found in ≥2 countries or regions.

The 208 isolates from Australia were grouped into 37 MTs, of which the 4 most prevalent represented 65.4% of the isolates: MT27, 13.5%, including 1 isolate from 1973 and the others from the 1990s to 2008; MT29, 21.6%, observed since 1972; MT70, 21.2%, 1996–2005, mostly since ACV introduction in 1997; and MT64, 9.1%, during 1989–2002. Prevalence trends of the 4 most common MTs were analyzed for 3 periods determined by vaccine type(s) in use: WCV (prior to 1997), the transition period of both WCV and ACV (1997–1999), and ACV only (2000 onward) (Figure 1). MT64 prevalence was steady over time. MT29 decreased while MT27 and MT70 increased. Trends in Australia for MT27 and MT29 were similar to those observed in the United Kingdom (*11*) and the Netherlands (*10*). D values were 0.86, 0.83, and 0.83 for WCV, transition, and ACV periods, respectively. This slight decrease in genetic diversity might indicate expansion of clones that are better adapted to ACV-induced immunity.

We typed 5 genes, the products of which are used in ACVs (*prn*, *ptxA, fim2, fim3,* and *fhaB*), using the method of Chan et al. (*12*) to assess the effect of the switch from WCV to ACV on prevalence of the 4 predominant MTs (MT27, MT29, MT64, and MT70) in Australia. Isolates from these MTs have the same *ptxA1* and *fhaB1* alleles but vary in the other 3 genes investigated (complete list available from authors). The predominant ACV used in Australia is from GlaxoSmithKline (GSK) (Research Triangle Park, NC, USA), which contains pertussis toxoid, filamentous hemagglutinin, and pertactin but no fimbriae (FIMs). The strain used for GSK ACV contains the alleles *prn1*, *ptxA2,* and *fhaB1* (*10*,*13*). However, ACV from Sanofi-Aventis (Pasteur, Lyon, France), which contains FIM2+3 in addition to pertussis toxoid, pertactin, and filamentous hemagglutinin with unknown allele types, is also licensed in Australia, complicating interpretation of variation in *fim* genes. On the basis of their frequencies and late appearance, *fim2-2* and *fim3-B* are not likely to be the vaccine alleles. A significant increase ($p<0.005$) of *prn2* (36% vs. 3%), *fim2-2* (34% vs.

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Table 1. Primers used in study of *Bordetella pertussis* clones Identified by multilocus variable-number tandem-repeat analysis

Primer name	Sequence, 5′ → 3′	Genome coordinates*	Concentration,† Mix	μM	Reference
BP-VNTR1-DF	VIC-CCTGGCGGCGGGAGACGTGGTGGTG	2194507	1	0.13	(*10*)
BP-VNTR1-DR	AAAATTGCGGCATGTGGGCTGACTCTGA	2194862	1		(*10*)
BP-VNTR2-BF	VIC-CGCGCCGCCTACGACCGCTATGG	2647550	2	0.08	(*10*)
BP-VNTR2-BR	CCCGCGCCGAAGATCTCGCCAAAGATAT	2647412	2		(*10*)
BP-VNTR3-BF	FAM-GCCTCGGCGAAATTGCTGAAC	2591464	2	0.23	(*10*)
BP-VNTR3-BR	GCGGGCGAGGAAACGCCCGAGACC	2591350	2		(*10*)
BP-VNTR4-CF	NED-CGTGCCCTGCGCCTGGACCTG	185211	2	0.08	(*10*)
BP-VNTR4-BR	GCCGCTGCTCGACGCCAGGGACAA	185000	2		(*10*)
BP-VNTR5-BF	PET-GAAGCCGGCCCACCCGAGCTCCAGGCTCTT	1005290	1	0.06	(*10*)
BP-VNTR5-BR	TGCCGGGTTTCGGCATCTCGATGGGATACG	1005177	1		(*10*)
BP-VNTR6-EF	FAM-CCAACGGCGGTCTGCTGGGTGGTC	2099525	1	0.06	(*10*)
BP-VNTR6-FR	CGCCGCCCGCTGCGCCGCTACC	2099315	1		(*10*)
VNTR7F2	PET-ATCAGGAAACCCACCACCACGCCGG	124402	2	0.08	This study
VNTR7R2	GTCACCAGCCCGCAGTACTGGCG	124585	2		This study
VNTR8F2	NED-TGGGTGTCTCCGTGATAGTGAGCACTTACAC	444776	1	0.19	This study
VNTR8R2	CTGGCGCAAAAACAGTAAGCCCGCACG	444981	1		This study

*Based on genome sequence position of the Tohama I strain; VNTR, variable-number tandem-repeat.
†Concentrations listed are for forward and reverse primers separately.

8%), and *fim3-B* (24% vs. 0%) was observed in the ACV period in comparison to the WCV period.

This increase of allelic frequency is better reflected in changes in antigenic profiles. MT27 has 3 profiles (*prn1, fim2*-1, *fim3*-A*; prn2, fim2*-1*, fim3*-A*;* and *prn2, fim2*-1, *fim3*-B). The first profile was seen once in the WCV period, whereas the other 2 first appeared in the WCV/ACV transition period and increased in frequency in the ACV period; the third profile, which differed by 2 alleles from the first, was more frequent. The appearance of *prn2* in the second profile and additional change from *fim3-A* to *fim3-B* in the third represent increases in prevalence of alleles absent from ACV. MT29 also has 3 profiles ([*prn1, prn2,* or *prn3*], *fim2*-1, *fim3*-A), which differ in *prn* only. Most MT29 isolates carry *prn3,* and the profile is prevalent in both WCV and ACV periods, with no obvious increase in non-ACV alleles.

MT70 and MT64 both have uniform allelic profiles (*prn1*, *fim2*-2, *fim3*-A and *prn1*, *fim2*-1, *fim3*-A, respectively)**.** However, MT70 (with *fim2-2,* not likely to be in ACV) increased significantly over the study period while MT64 with all alleles likely to be in ACV remained steady. Overall, the frequency of MT27 and MT70, with non-ACV alleles, increased significantly ($p<0.0001$) and correlated with the introduction of ACV, suggesting that antigenic changes could be driven by selection pressure.

The 2 MTs predominant in Australia were also prevalent in other countries and possibly have a global distribution. MT27 (18% of isolates) was found in 8 countries and MT29 (17% isolates) in 5. However, absence of an MT in a country might result from the small samples used**.** MT27 and MT29 were the most common types in the Netherlands (*10*) and the United Kingdom (*11*). MT10, MT64, MT70, MT84, and MT186 were also relatively common. MT10 and MT186 were found predominantly in Japan, although each had been found elsewhere, in China (1957) and Hong Kong (2002), respectively. MT64 was predominantly from Australia with 1 isolate from Japan, and MT70 was only found in Australia. However, all of these frequent MTs (except MT186) have been observed before. MT10 was frequent in the United Kingdom in the prepertussis vaccine era, while MT70 was common during 1998–2001 (*11*).

Nine isolates, including Tohama I, indentified in samples of pertussis strains collected during the 1920s–1950s

Table 2. Diversity of variable-number tandem-repeat analysis loci *Bordetella pertussis* isolates*

Locus	No. repeats	Global (this study)		Australia (this study)		The Netherlands (*10*)	
		No. alleles	*D*	No. alleles	*D*	No. alleles	*D*
VNTR1	2–12	6	0.58	4	0.63	7	0.26
VNTR2	2–5	4	0.02	2	0.01	3	N/A
VNTR3a	2–8	4	0.40	4	0.43	10	0.18
VNTR3b	0–10	5	0.21	5	0.21	4	0.15
VNTR4	2–9	7	0.34	5	0.24	8	0.21
VNTR5	3–9	6	0.20	4	0.19	7	0.18
VNTR6	2–11	8	0.72	5	0.70	8	0.60
VNTR7	3–4	2	0.01	1	0.00	NA	NA
VNTR8	2–4	3	0.16	2	0.03	NA	NA

**D*, Simpson index of diversity; VNTR, variable-number tandem-repeat; NA, not applicable.

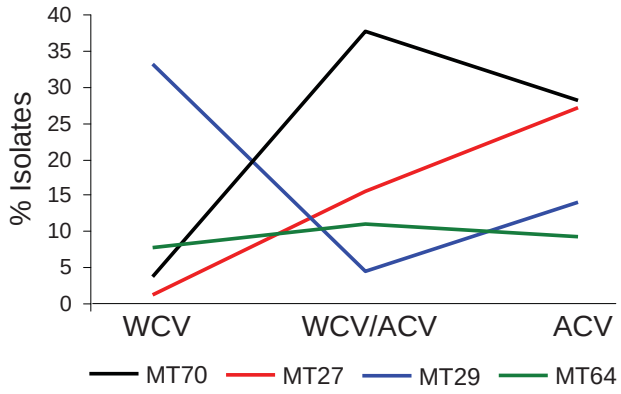

Figure 1. Temporal trends of predominant multilocus variable-number tandem-repeat analysis (MLVA) types in Australia. Isolates of 4 major MLVA types (MT70, MT27, MT29, and MT64) obtained in Australia were divided into 3 periods: whole cell vaccine (WCV) (before 1997), transition from WCV to acellular vaccine (ACV) (1997–1999) and ACV (2000 onward).

from 5 countries (China, France, Japan, United Kingdom, and United States) were distributed among 7 MTs: MT10, MT12, MT75, MT83, MT127, MT205, and MT206, 2 of which were also represented among recent strains: MT10, 6 isolates from Japan 1989–2007; and MT75, 1 isolate from France in 1993. The remaining 5 MTs were either unique or shared only among the 9 early isolates.

MLVA data were used to construct a minimum spanning tree (MST) (Figure 2). The 66 MTs were grouped into 2 clonal complexes and 9 singletons. Most MTs (54 of 66) belong to 1 clonal complex and 3 (MT186, MT187, and MT194) belong to another. Relationships between singletons with multiple allelic differences are not robust because they can be connected to other nodes equally. Thus, the MST cannot be rooted to infer the direction of change. Two internationally predominant MTs (27 and 29) are closely related with 1 allele difference. MT10, prevalent in Japan, is also closely related to MT29, with 1 allele difference. MT29, first isolated in the prevaccine era in the United Kingdom (*11*), has the highest number of SLVs and was found over 4 continents, which suggests that it arose early. Because MT10 and MT27 have a high frequency of SLVs, both likely emerged quite early. MT10 was isolated as early as 1957 in China and MT27 in 1950 in the Netherlands (*10*). Two high-frequency MTs (MT64 and MT70) were found in Australia only recently, with few SLVs, and may have contributed to the resurgence of pertussis in Australia.

Conclusions

Analysis of 208 isolates from Australia and representative isolates of common pulsed-field gel electrophoresis types from Canada, Japan, Finland, and the United States identified 6 predominant MTs (clones). Two (MT27 and MT29) were distributed worldwide, while 4 (MT10, MT64, MT70 and MT186) predominated in specific countries. Several MTs have persisted over long periods, including 3 that have circulated for at least half a century. Typing of genes encoding ACV antigens showed that use of ACV may have driven antigenic changes of 2 MTs now predominant in Australia.

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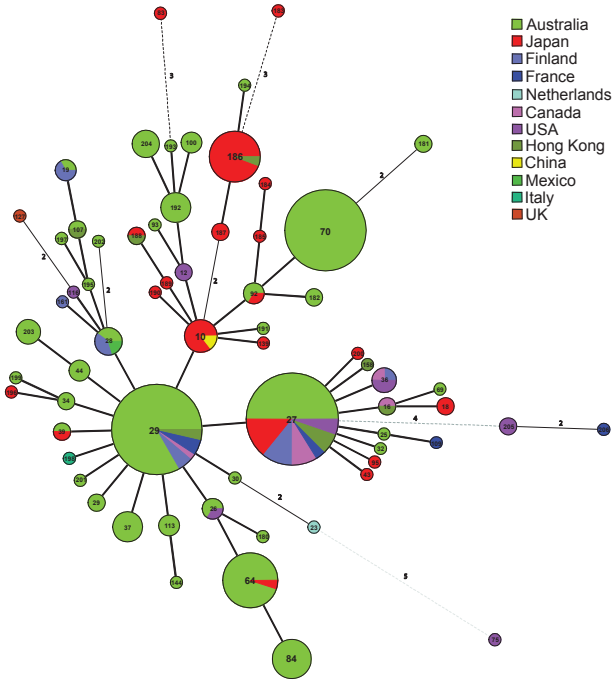

Figure 2. Minimum spanning tree (MST) of multilocus variable-number tandem-repeat analysis (MLVA types for global *Bordetella pertussis* isolates. The MST produced in Bionumerics (Applied Maths, Kortrijk, Belgium) used categorical coefficient and the eBURST priority rule of the highest number of single-locus changes for the clustering. Each circle represents an MLVA type with the type number in the circle. Thick lines, types differing by a single MLVA locus; thin lines, double-locus variants; dotted lines, 2 types differing by >2 MLVA loci. The size of the circle reflects the number of isolates with a given MLVA type. The color codes for country of origin are shown, and pie charts within a circle are used to indicate the proportion of isolates.

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Plasmodium falciparum Malaria, Southern Algeria, 2007

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An outbreak of *Plasmodium falciparum* malaria occurred in Tinzaouatine in southern Algeria in 2007. The likely vector, *Anopheles gambiae* mosquitoes, had not been detected in Algeria. Genes for resistance to chloroquine were detected in the parasite. The outbreak shows the potential for an increase in malaria vectors in Algeria.

Outbreaks of malaria in southern Algeria have been observed for many years, including a major epidemic in Dajnet in 1928–1929 (*1*). Most (>90%) documented cases were attributed to *Plasmodium falciparum* (*2*); *Anopheles sergenti* and *An. multicolor* mosquitoes were incriminated as potential vectors (*3*). The Sahara Desert has been regarded as an effective barrier against northward expansion of *An. gambiae* mosquitoes, the main malaria vector in Africa, into Algeria. However, this mosquito has been detected near the Algeria–Mali border (*4*).

In recent years, marked changes in the environment and the economy of southern Algeria have occurred (exploitation of underground water resources, growth of the human population in several oases, and development of a transport infrastructure). The new Trans-Saharan Highway, which links Algeria and West Africa, is a potential route for introduction of tropical vectors and parasites into southern Algeria (*2*,*5*).

In November 2007, a total of 26 autochthonous cases of *P. falciparum* malaria were detected in Tinzaouatine, a village in Algeria near the Algeria–Mali border. We present results of a parasitologic and entomologic study conducted during the outbreak and discuss the potential for establishment of vectors and *P. falciparum* malaria in Algeria.

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The Study

Tinzaouatine (altitude 620 m, 19.95°N, 2.96°E, population ≈12,000) is a village near the Mali border, ≈2,000 km south of Algiers and 578 km southeast of Tamanrasset. Most of its inhabitants are nomadic Tuareg. The climate is arid (annual mean temperature 27°C, range 17°C–33°C, <100 mm rain/year; Office Nationale de Météorologie, Algiers, Algeria). Precipitation is associated with the West African monsoon and restricted to a short period (June–September). The Tinzaouatine River, which is dry for most of the year, occasionally floods. After flooding, receding water results in abundant pools (*gueltas*) that are ideal breeding sites for anopheline mosquitoes. Livestock (mostly sheep and goats) are common in the region. However, no agricultural activity takes place and no irrigation system exists.

During a 2-week period in December 2007, adult mosquitoes were collected by morning indoor spraying in 4 houses and 3 nomad tents (2×/week), human landing catches (2 nights/week), and CDC light traps and mouth aspirators in resting sites (at night). Adult sampling was conducted in dwellings of persons with cases of malaria. Human landing catches were made on 2 adult volunteers from the medical research team from 8:00 PM to 6:00 AM. Larvae and pupae were collected by dipping into 2 mosquito-positive pools (1× over a 3-hour period). Adult mosquitoes derived from pupae were identified by using morphologic keys (*6*) and genotyped by rDNA PCR to determine species within the *An. gambiae* complex (*7*).

Eleven pools were tested for anopheline larvae. However, many had been treated with insecticide. Larvae of anophelines and other species were collected from 2 gueltas (area 30 m^2 and 200 m^2, respectively). A total of 123 anopheline larvae were reared into adults (35 males and 12 females hatched). All specimens were of the Mopti (M) molecular form of *An. gambiae sensu stricto* mosquitoes. Use of entomologic controls at the same site in 2008 confirmed that the unique anopheline species present in this area was *An. gambiae sensu lato*. No adult mosquitoes were captured, probably because of insecticide spraying during the period of sampling to control the outbreak and because of a temperature <10°C at night.

Clinical diagnosis of malaria was made at the health center in the village. A total of 1,468 samples were examined by microscopy during the outbreak. Twenty-six patients (11 female and 15 male, age range 1–43 years) who had fever, chills, and rigor had samples positive for *P. falciparum*. None of these patients had traveled outside Tinzaouatine before the outbreak, which indicated that these cases were autochthonous.

All patients were treated with chloroquine (10 mg/kg/day for 2 days and 5 mg/kg for 3 days). Clinical resistance to chloroquine was not reported and no deaths occurred. Informed consent was obtained from each patient or adult guardian of children enrolled in this study at the time of blood collection.

Ten samples were chosen for molecular study; 8 were from patients positive by microscopy and 2 were from patients with malaria symptoms negative for *P. falciparum* by microscopy. Samples were processed by placing a drop of blood on filter paper. Molecular analysis was conducted according to the protocol described by Snounou et al. (*8*). Molecular screening by real-time PCR was used to detect mutations in the *P. falciparum* dihydrofolate reductase (*dhfr*), dihydropteroate synthase (*dhps*), and chloroquine resistance transporter (*crt*) genes, which are involved in *P. falciparum* drug resistance (*9*). Molecular analysis results of 5 PCR-positive blood samples showed the *pfcrt* 76T and the *dhfr* 108N mutations, and 4 showed the quadruple mutation (*dhfr* 51I, 59R, 108N and *dhps* 436A). No mutations were detected in the *dhps* 540 codon. We also identified a *P. falciparum* isolate with a unique sextuple drug resistance profile ($Y_{86mdr1}/T_{76crt}/I_{51dhfr}/R_{59dhfr}/N_{108dhfr}/A_{436dhps}$).

Although all patients were treated with chloroquine and despite our evidence of polymorphisms in genes linked to chloroqunie resistance, no clinical failures were observed. Polymorphisms in the *crt* gene are strongly associated with chloroquine resistance. Involvement of the *mdr1* gene in chloroquine resistance has been challenged, but variation at codon 86 of this gene modulates resistance to chloroquine (*10*).

Conclusions

An average of 300 cases of malaria is recorded in cities in southern Algeria every year, mostly in Tamanrasset and Adrar. Parasites are introduced by infected humans; >90% of cases originate in Mali and Niger (*11*). Several autochthonous infections have been reported in Tinzaouatine (Table). We suggest that introduction of malaria into this area likely reflects the highly mobile nature of local populations and associated travel to or from areas endemic for malaria. Interethnic conflicts in northern Mali have also increased the displacement of populations toward Algeria.

Table. Cases of autochthonous malaria, Tinzaouatine, Algeria, 1999 and 2003–2005*

Species	No. case-patients			
	1999	2003	2004	2005
P. falciparum	4	11	1	0
P. vivax	0	1	3	1
P. malariae	0	0	0	0
P. ovale	0	0	0	0

*All case-patients were positive for *Plasmodium* spp. by microscopy and treated with chloroquine.

An. gambiae mosquitoes in Algeria probably originated in Mali. Algeria has borders with Mali and Niger, countries where *An. gambiae sensu lato* mosquitoes are present (*4*). The most likely mode of introduction of *An. gambiae* mosquitoes into Algeria is passive transport by vehicles and trucks because considerable traffic moves across its borders (*12*). An alternative hypothesis is that mosquitoes were carried by wind from breeding sites in southern Mali, a mode of dispersal that has been described for other species of mosquitoes (*13*).

An. gambiae mosquitoes found in Algeria were of the M molecular form. Current knowledge of genetics and distribution of *An. gambiae sensu lato* mosquitoes (*14,15*) suggests that this species is probably of the chromosomal M form, which is adapted to an arid climate. Unfortunately, chromosomal inversions in mosquitoes found in Algeria could not be assessed because of lack females at the half-gravid stage (during blood digestion and egg development). However, conditions in 2007 were probably favorable for establishment of this species because the infested area had experienced unusual heavy rainfall, particularly in November (Figure). Moreover, the location of this species ranges from the Sahel in West Africa to rain forests in equatorial regions (*14*).

We cannot predict whether this highly effective vector will become established in southern Algeria, with an accompanying risk for establishment of malaria, or whether this region will be a source of vectors and parasites that could threaten the rest of Algeria. The bionomics of this species and its ability to survive the long hot and dry season are among several factors that need to be investigated.

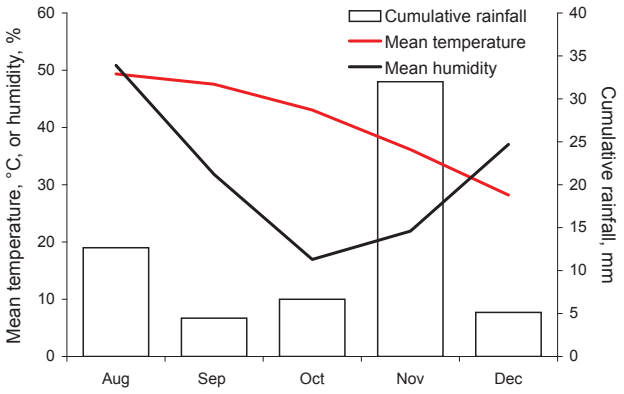

Figure. Climatic data for Tinzaouatine, Algeria, August–December 2007 (Algerian Ministry of Meteorology).

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Pandemic (H1N1) 2009 Outbreak on Pig Farm, Argentina

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In June–July 2009, an outbreak of pandemic (H1N1) 2009 infection occurred on a pig farm in Argentina. Molecular analysis indicated that the virus was genetically related to the pandemic (H1N1) 2009 influenza virus strain. The outbreak presumably resulted from direct human-to-pig transmission.

Swine influenza viruses (SIVs) occur worldwide; they usually cause asymptomatic infection but occasionally cause outbreaks of varying intensity (*1*,*2*). In North America, the landscape of swine influenza has changed substantially since the late 1990s, when human (H3N2) viruses were first isolated from swine and triple reassortant viruses carrying influenza genes (TRIG) of avian, swine, and human origin and showing great reassortment flexibility emerged concurrently. TRIGs of influenza subtypes H3N2, H1N1, H3N1, and H1N2 appear to be circulating in swine in North America. Coincidentally, 1 of these TRIG viruses led to emergence of the pandemic (H1N1) 2009 virus (*3*). Testing by the Canadian Food Inspection Agency identified pandemic (H1N1) 2009 virus in a swine herd in Alberta (*4*). The pigs may have been exposed to the virus by a farm worker who returned from Mexico with influenza-like symptoms (*5*). Affected pigs showed clinical signs of infection with SIV (*5*).

During the past influenza season (April–September 2009) in the Southern Hemisphere, pandemic (H1N1) 2009 virus predominated in humans and accounted for >90% of influenza cases in humans in Argentina. In Argentina, seroepidemiologic analyses of pigs on 17 farms showed that ≈41% of pigs had antibodies against H1 and H3 (*6*). Vaccines against SIV are not licensed for use in Argentina.

We describe the epidemiology, clinical outcome, pathology, and persistence of pandemic (H1N1) 2009 infection on a pig farm 103 km from Buenos Aires. Molecular analysis indicated that the virus was genetically related to the pandemic (H1N1) 2009 virus strain and indicated no evidence of further reassortment with any other influenza strains. The outbreak presumably resulted from direct human-to-pig transmission at the peak of the pandemic in Argentina.

The Study

The pig farm, a closed farrow-to-finish 1-site operation in Buenos Aires province, had 519 sows. Pig death rate from farrowing to slaughter remained at 9.5% before and after the outbreak. Ten days before the outbreak, the farm manager and his wife reported clinical signs of influenza in themselves.

On June 15, 2009, nursery barn pigs >40 days of age showed clinical signs, e.g., cough, dyspnea, fever, nasal discharge, and inappetence. Illness affected 30% of nursery pigs. On June 17, pigs in 8 growing and fattening barns showed similar clinical signs; 4,000 (15%) pigs were affected). Clinical signs lasted for 1 week; on June 22, resolution of clinical signs was evident, with cough index <2% in growing and fattening.

At the onset of the outbreak, 5 clinically affected pigs were submitted for postmortem examination. Tissue samples from nose, trachea, and lungs were fixed in 10% buffered formalin and processed for routine histopathologic examination. We conducted immunohistochemical (IHC) analysis using antinucleoprotein influenza A monoclonal antibody, isotype MSIg2a (Chemicon International Inc., Temecula, CA, USA) according to Vincent et al. (*7*). In addition, we obtained 30 serum samples from nursery, growing, and fattening pigs.

All pigs had cranioventral lung consolidation on 5%–60% of the total surface. Two of 5 pigs had distinctive, scattered, dark-red foci of lobular consolidation (chessboard-like) (Figure, panel A). In 3 pigs, we observed serofibrinous polyserositis.

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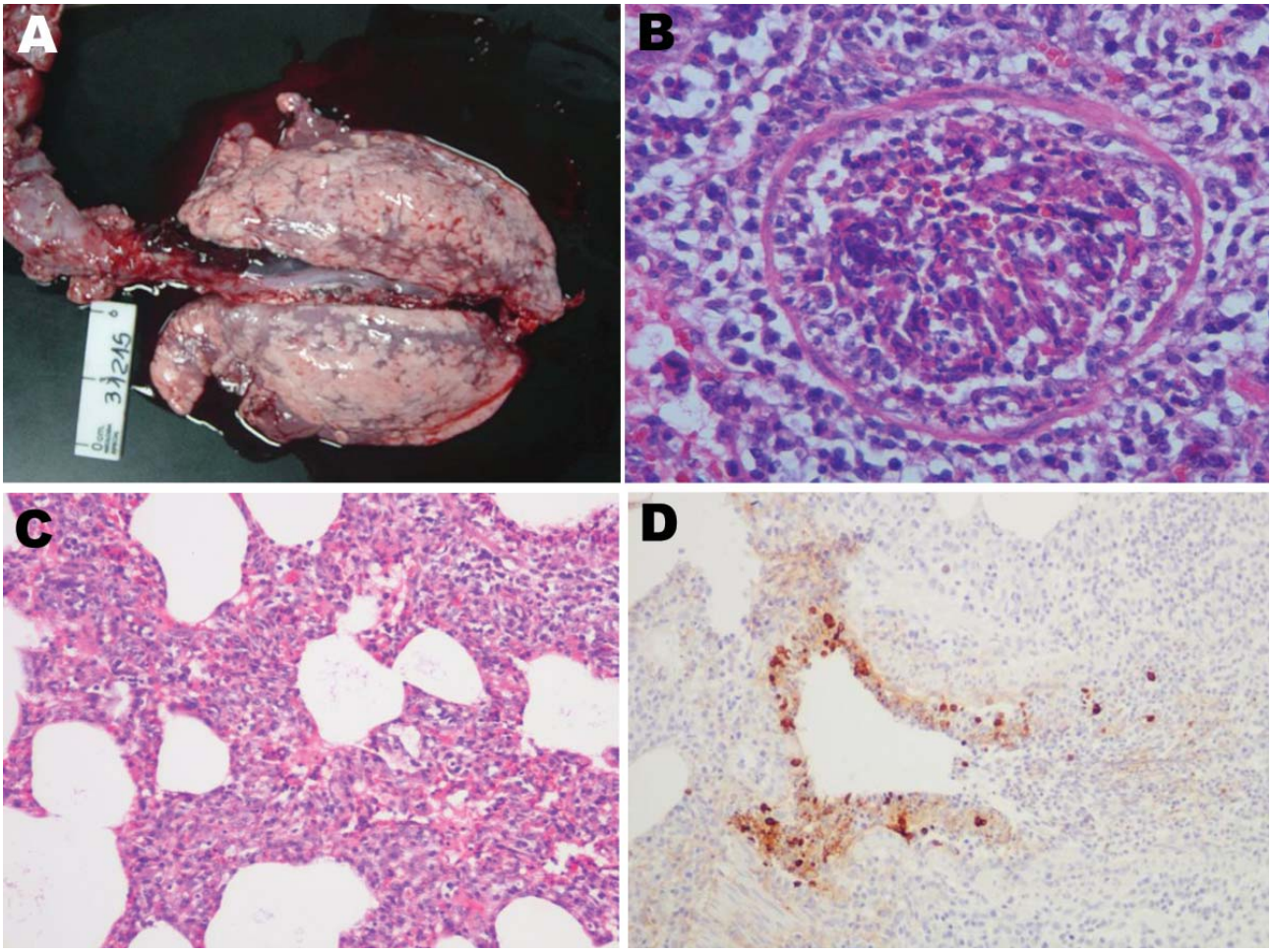

Figure. Postmortem samples from clinically affected pigs, Argentina, 2009. A) Macroscopic lung lesions with distinctive scattered dark-red foci of lobular consolidation (chessboard-like) in all lobes. B) Severe necrotizing bronchiolitis with partially denuded epithelia. Hematoxylin-eosin stain; original magnification ×400. C) General view of the alveolar walls showing moderate interstitial thickening by leukocytes. Hematoxylin-eosin stain; original magnification ×200. D) Bronchioli. Positive-labeled nuclei and cytoplasm of bronchial epithelial cells and mononuclear cells in the alveoli. 3,3′-diaminobenzidine counterstain with hematoxylin stain; original magnification ×200.

Four pigs had severe necrotizing bronchiolitis. Small and medium bronchioles were plugged with neutrophils cellular debris and exudates. Affected airways were denuded or lined with flat epithelium (Figure, panel B). The adjacent alveolar walls were infiltrated and thickened in a lobular fashion by mononuclear cells (Figure, panel C). In other pigs, the alveolar lumen was filled with macrophages, neutrophils, and fibrinous exudates. We observed a positive IHC reaction in bronchiolar epithelia only, which showed severe inflammatory changes (Figure, panel D).

We processed 5 bronchial swabs and lung samples from postmortem-examined pigs for real-time reverse transcription–PCR (rRT-PCR) and for virus isolation in MDCK cells. Viral RNA was extracted from swab suspension and from culture supernatant (Total RNA Isolation Kit; Applied Biosystems, Foster City, CA, USA), and viral cDNA was synthesized. We tested the cDNA for influenza type A by rRT-PCR directed to the matrix (M) gene, as previously reported (*8*), and tested for pandemic (H1N1) 2009 virus by rRT-PCR using the protocol released by the Centers for Disease Control and Prevention (CDC) on April 30, 2009 (revision 1) (www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html).

Next, we conducted a cross-sectional study of 60 nasal swabs from pigs of 3 categories (i.e., nursery, growing, fattening) on June 25, July 2, July 10, and July 31. All these samples were negative. Also, 120 bronchial swabs were sampled at slaughter on June 30, July 3, and July 7; 1 sample from June 30 and 5 from July 3 were positive, but thereafter, all bronchial swabs were negative. A serologic survey was conducted of 214 fattening pigs on June 30 and every week thereafter by using ELISA (HerdChek Swine Influenza H1N1 Antibody Kit; IDEXX Laboratories, Westbrook, ME, USA).

All postmortem samples were positive for subtype (H1N1) according to both rRT-PCR protocols, and cycle threshold values ranged from 16 to 27 cycles according to the CDC protocol. Nasal samples from pigs in the cross-sectional study were negative. However, 5% of the bronchial swabs sampled from clinically healthy pigs at slaughter were positive according to both rRT-PCR protocols. At the onset of the outbreak, all pig serum samples were negative for swine (H1N1) influenza virus. However, 98% of serum samples from slaughter pigs were positive 15 days later.

Cytopathic effects in MDCK cells were noted on first passage at 72 h postinoculation, and this virus was characterized by sequencing as pandemic (H1N1) 2009. All genome segments were fully amplified by RT-PCR (*9*) and directly sequenced from the isolate in MDCK. Regions corresponding to the entire open reading frame of hemagglutinin (HA), nucleoprotein, neuraminidase (NA), M1, and nonstructural and partial polymerase base (PB) 2 (nt 580–1140), PB1 (nt 390–990), and polymerase acid (PA) (nt 720–1230) segments were obtained by using appropriate set of primers and submitted to GenBank (accession nos. CY044256–CY044260 and CY047749–CY047751). High (>99.99%) nucleotide identity was found between these sequences and pandemic strain A/California/04/2009 (H1N1) and A/swine/Alberta/OTH-33-8/2009 (H1N1) (Table).

Conclusions

Young pigs are susceptible to pandemic (H1N1) 2009 as reported in Canada (*4*,*5*). Recently, experimental infections showed that the pandemic (H1N1) 2009 virus induced mild clinical signs, virus shedding, and gross and histopathologic lesions similar to those caused by SIV (*10*,*11*). Successful cross-species transmission usually requires a period of adaptation to the new host (*12*). However, phylogenetic analyses of pandemic (H1N1) 2009 virus showed that the HA and NA glycoproteins arose from the classical swine (H1N1) and avian-like Eurasian swine (H1N1) lineages, respectively (*3*), which might explain the high susceptibility and transmissibility among pigs.

Table. Nucleotide identity percentage of swine influenza viruses in a study of a pandemic (H1N1) 2009 outbreak at a pig farm, Argentina, June–July 2009

Genome segment*	A/California/04/2009 (H1N1)	A/swine/Alberta/OTH-33-8/2009 (H1N1)
1 (PB2)	100	99.994
2 (PB1)	100	99.998
3 (PA)	100	99.998
4 (HA)	99.997	99.994
5 (NP)	99.996	99.993
6 (NA)	99.998	99.995
7 (M1)	99.997	99.996
8 (NS)	99.999	99.994

*PB, polymerase base; PA, polymerase acid; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural protein.

From an epidemiologic standpoint, the 25%–30% morbidity rate with few deaths agreed with the clinical presentation of SIV (H1N1) (*1*,*13*) and was similar to the rates found in the outbreak in Canada (*4*,*5*). Clinical signs lasted 1 week. However, 5% of the bronchial swabs taken 15–18 days after onset of the outbreak from slaughter-weight pigs showed positive results. Virus shedding, detected by rRT-PCR, from nasopharyngeal samples peaked at 4 days postinoculation and ceased at ≈11 days postinoculation.

Epithelial cell necrosis, sloughing, and neutrophil infiltration with total or partial obstruction of bronchioles is the hallmark of SIV infection (*14*) and was distinctive for influenza A virus (H1N1). However, IHC results were strongly positive for virus antigen in only those bronchioles that showed severe bronchiolitis. Our findings from field observation confirm findings from experimental studies with pandemic (H1N1) 2009 virus (*11*).

The National Veterinary Services Laboratory has established a farm biosecurity program to survey the infection. The program includes movement restrictions and clinical surveillance of farms within 3-km of the index case. No other farm had clinically ill pigs. The farm was allowed to move animals to slaughter after the nasal swabs of those animals tested negative by rRT-PCR with the CDC protocol.

The suspected human source of infection was not confirmed. This outbreak supports the belief that influenza cross-species transmission may occur between pigs and humans and vice versa.

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Sin Nombre Virus Infection in Field Workers, Colorado, USA

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We report 2 cases of Sin Nombre virus (SNV) infection in field workers, possibly contracted through rodent bites. Screening for antibodies to SNV in rodents trapped in 2 seasons showed that 9.77% were seropositive. Quantitative real-time PCR showed that 2 of 79 deer mice had detectable titers of SNV RNA.

Hantaviruses are rodent-borne viruses that in the Americas have been implicated as the causative agents of hantavirus cardiopulmonary syndrome (HCPS) (*1*). In North America, Sin Nombre virus (SNV) is responsible for most cases of HCPS, and the deer mouse (*Peromyscus maniculatus*) is its main reservoir. Since the first reported outbreak of the disease in 1993 in the southwestern United States, rodent serologic surveys have confirmed that SNV is present through most of the range where deer mice exist, including Colorado (*2*,*3*). We report 2 cases of SNV infection in field workers in Colorado, who were infected with the virus despite protection with a powered air-purifying respirator. We also performed a serologic survey of wild rodents in the presumptive areas of exposure and quantitative real-time PCR analyses of blood samples from deer mice identified as seropositive.

The Study

In this study of human infections by SNV in Boulder County, Colorado, we identified 2 patients who had trapped rodents for ecologic studies. On June 14, 2005, a 24-year-old man was admitted to Boulder Community Hospital with fatigue, headache, fever, and thrombocytopenia (70,000 platelets/μL) but without cardiorespiratory compromise. A strip immunoblot assay identified immunoglobulin (Ig) M and IgG against SNV N and Gn proteins. On July 6, 2005, a 22-year-old IgM- and IgG-seropositive woman was admitted to Boulder Community Hospital with fever and dyspnea; she subsequently experienced bilateral lung infiltrates and thrombocytopenia (116,000 platelets/μL). She required oxygen supplementation but recovered almost completely by July 11. She reported performing fieldwork in the same period as did patient 1 but with no overlap among the sites (the distances between sampling sites where the 2 field workers most probably contracted their infections ranged from 6.4 to 9.8 km). Both patients engaged in field activities involving manipulating traps and rodents in areas where deer mice were seropositive for SNV (Table).

Along with the 2 patients, another 15 field workers were surveyed to assess possible exposures to SNV. Ninety-five questions were asked involving, among others, contacts with rodents and use of personal protective measures and equipment. Most (83.3%, 14/15) reported previous experience with rodents in the field; all workers were required to wear nitrile gloves and use a powered air-purifying respirator when handling animals. No differences in risk exposure to contract hantavirus were evident between infected and noninfected persons. Six persons reported having been bitten ≥1 times by rodents, including both case-patients. Patient 2 was bitten twice, with 1 bite resulting in bleeding despite the worker's use of nitrile gloves. Patient 1 reported being bitten by a vole (*Microtus* sp.) on June 2, and patient 2 was bitten by 2 deer mice on June 14. Their wounds were treated by immediate cleaning and bandaging. Patient 2 also applied an antimicrobial ointment before bandaging her bleeding wound.

We hypothesized that the workers might have been exposed to a subset of rodents with unusually high titers of SNV. Therefore, we resampled sites in Boulder and Jefferson counties in Colorado (where the field workers were infected) during August–September 2005. A total of 44 sites were sampled during both trapping periods by using live traps (H.B. Sherman Traps, Tallahassee, FL, USA) in grids for 4 consecutive nights. Prairie dogs (*Cynomys ludovicianus*) were also trapped at a subset of these study sites during June–July 2005 with live traps (Tomahawk Live Traps, Tomahawk, WI, USA). A total of 1,868 animals from 10 mammalian species were captured during both trapping periods (Table).

We screened blood samples by strip immunoblot assay for antibodies against SNV N protein (*4*). Four rodent species yielded positive samples from 197 blood samples. Deer mice showed the highest abundance of seropositive samples, although harvest mice (*Reithrodontomys megalotis*), which carry El Moro Canyon virus, had higher seroprevalence. Two (2.5%) of 81 hispid pocket mice (*Chaetodipus hispidus*) were also positive but are unlikely to play an epidemiologic role. Small mammal capture frequencies

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Table. Mammal species, abundance, seropositivity, and Sin Nombre virus RNA quantification during May–June and August–September 2005, Boulder, Broomfield, and Jefferson counties, Colorado, USA*

Species (common name)	No. animals		No. seropositive		Antibody prevalence, %		SNV titers
	May–Jun	Aug–Sep	May–Jun	Aug–Sep	May–Jun	Aug–Sep	
Chaetodipus hispidus (hispid pocket mouse)	23	58	0	2	0	3.44	
Cynomys ludovicianus (black-tailed prairie dog)	171	33	0	0	0	0	
Microtus ochrogaster (prairie vole)	26	11	0	1	0	9.1	
Microtus pennsylvanicus (meadow vole)	10	1	2	0	20	0	
Mus musculus (house mouse)	1	2	0	0	0	0	
Neotoma mexicana (Mexican woodrat)	3	0	0	0	0	0	
Peromyscus maniculatus (deer mouse)	711	780	105	82	14.77	10.5	Animal 1050, 520.8 copies/mL; animal 2404, 87.45 copies/mL
Reithrodontomys megalotis (western harvest mouse)	8	21	1	4	12.5	19.0	
Spermophilus tridecemlineatus (thirteen-lined ground squirrel)	3	4	0	0	0	0	
Sylvilagus audubonii (Audubon's cottontail)	1	1	0	0	0	0	

*SNV, Sin Nombre virus.

varied during the 2 sampling periods; seroprevalence for pocket mice, prairie voles, and harvest mice increased, and that for meadow voles (*M. pennsylanicus*) decreased. Seroprevalence among deer mice was higher during May–June (when the field workers contracted their infections) than in August–September.

We performed TaqMan (Applied Biosystems, Foster City, CA, USA) quantitative real-time PCR on a subset of 79 (of 187) samples from deer mice that had detectable antibodies to SNV N antigen. The samples selected for PCR analysis were those for which the volume of blood was deemed sufficient (>25 μL) to carry out a satisfactory RNA extraction. We chose 25 μL as the minimal amount for detecting SNV small segment RNA by nested reverse transcription–PCR on the basis of a spiking experiment in which 5 μL of lung homogenate from an infected deer mouse had been added to 20 μL of blood from an uninfected deer mouse, resulting in a positive finding. The equivalents of 10-μL aliquots of total blood RNA (RNeasy Mini Kit; QIAGEN, Valencia, CA, USA) were subjected to quantitative real-time PCR with primers, probes, and PCR conditions as described (*5*). We detected only low levels of SNV in the blood of 2 of the 79 seropositive deer mice tested (Table). This low number of samples with detectable SNV RNA (2.53%) is congruent with previous findings reporting undetectable levels of SNV RNA in blood using quantitative real-time PCR (*6*).

Conclusions

The primary mode of hantavirus transmission to humans is through rodent excreta and secretions through the aerosol route (*7*). Although indoor exposure in poorly ventilated buildings has been reported as a major factor for contraction of HCPS, our survey supports the possibility that the 2 patients contracted SNV outdoors and that, in at least in 1 case, a rodent bite was the proximate vehicle for transmission of SNV to the field worker. This route of transmission is uncommon with only few examples reported (*8–10*). The fact that patient 1 was bitten by a vole and not by a deer mouse does not necessarily exclude transmission of SNV by that route. Voles are not known to transmit SNV, but there have been repeated instances of vole-associated hantaviruses being carried by sigmodontine rodents (*11*). Thus, sigmodontine-borne hantaviruses might also replicate productively in voles. Although the power of this survey is limited by small sample size, we believe that our finding are potentially useful and suggest that increased attention be devoted toward avoiding rodent bites among the handlers of wild rodents in regions where hantaviruses occur. Although both workers sustained rodent bites, 1 by a known SNV carrier and 1 by another rodent species, one should remain open-minded about the actual route of infection, which might still be through an airborne route rather than through bites in either case.

Our results suggest that detecting SNV RNA of sufficient magnitude (>80 copies/mL) to score as positive in TaqMan assays might be uncommon in the natural reservoir. Therefore, high loads of SNV RNA might not be a major factor in virus transmission in the wild. Alternatively, SNV might cause only a brief RNA viremia in wild deer mice (*12*), and possibly the small number of real-time PCR–positive deer mice represents those animals that un-

derwent recent seroconversion. This phenomenon has also been observed with other rodent borne-hantaviruses (*13*). Alternatively, or in addition, the small number of mice found to have quantifiable viral RNA in this study might be a consequence of physiologic events (such as viral recrudescence) (*14*), which result in intermittent detection of viral RNA in blood, a phenomenon that might be shared by other agents of hemorrhagic fevers (*15*).

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Pandemic (H1N1) 2009 Cases, Buenos Aires, Argentina

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To determine clinical and virologic characteristics of pandemic (H1N1) 2009 in Buenos Aires, Argentina, we conducted real-time reverse transcription–PCR on samples from patients with influenza-like illness, June 11–30, 2009. Of 513 patients tested, 54% were positive for influenza virus subtype H1N1. Infection rate was lowest for patients ≥60 years of age.

A novel influenza A (H1N1) virus emerged in mid-April 2009 and spread rapidly among humans worldwide (*1*,*2*). The virus, pandemic (H1N1) 2009 virus, is a unique combination of gene segments from North American and Eurasian swine lineages and ancestral genes derived from avian species and humans (*3*,*4*). In Buenos Aires, Argentina, the first laboratory-confirmed case of pandemic (H1N1) 2009 was documented May 16, 2009, in a patient who had traveled to the United States. This index case seeded an elementary school outbreak in Buenos Aires, and, within days, several schools reported increasing numbers of cases. The public health response included closure of schools with laboratory-confirmed influenza subtype H1N1 cases, voluntary self-isolation and oseltamivir treatment of suspected and confirmed patients, and recommended chemoprophylaxis for contacts. Despite these interventions, cases increased among the school-age and general population (*5*).

In early June, the number of confirmed cases increased among young adults in Buenos Aires. In addition, cases more severe than those previously seen in school students were being identified among young adults and pregnant women, some of whom required hospitalization in intensive care units. On June 11, 2009, the World Health Organization declared a phase 6 pandemic of this subtype H1N1 virus (*6*).

Argentina's National Reference Laboratory (Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud, ANLIS, "Dr. Carlos G. Malbrán") was initially designated as the only approved laboratory for diagnosing pandemic (H1N1) 2009 virus infections. After the rapid increase in the number of cases and the elevation of the pandemic alert level, the virology laboratory at Centro de Educación Médica e Investigaciones Clínicas (CEMIC) University Hospital in Buenos Aires also began diagnosing infections. This report describes clinical and virologic findings for the initial cases of pandemic (H1N1) 2009 diagnosed at CEMIC.

The Study

Study patients were enrolled from June 11 through 30, 2009, a time that corresponds to the end of autumn and the beginning of winter in the southern hemisphere. Patients seeking care at CEMIC University Hospital and other healthcare providers in Buenos Aires (Federal District) were enrolled if they met the criteria for an influenza-like illness. During the study period, respiratory samples from 513 patients were submitted to CEMIC's virology laboratory for pandemic (H1N1) 2009 testing.

Combined nasal and pharyngeal swab specimens or nasopharyngeal or tracheal aspirates were obtained from patients and shipped to CEMIC in viral transport media. Epidemiologic and demographic parameters and information on clinical signs, underlying disease, chest radiographs, and medication use were obtained for each patient.

A laboratory diagnosis of influenza infection was determined by real-time reverse transcription–PCR (RT-PCR), by using the Centers for Disease Control and Prevention's protocol, on a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) (*7*). The QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) was used to extract RNA. Statistical analyses were performed by using χ^2 and Fisher exact tests.

Of 513 patients, 275 (54%) had positive results for influenza subtype H1N1 by RT-PCR (Table 1). Of these 275 case-patients, 197 (72%) were outpatients and 78 (28%) were hospital inpatients.

Pandemic (H1N1) 2009 was detected most frequently among patients <5 years of age (59%), followed by those 19–59 years of age (57%). The lowest prevalence (37%) was among those ≥60 years of age; the difference in prevalence among those in this group and those in the groups <5 and 19–59 years of age was significant ($p = 0.019$ and $p = 0.003$, respectively) (Table 1). The highest percentage of hospitalizations occurred among patients <5 years of age (51%), followed by those ≥60 years of age (36%). Among patients 19–59 years of age, 24% required hospitalization (Table 1).

The first case-patient with pandemic (H1N1) 2009 was admitted to CEMIC on June 4, 2009. The patient, a 29-year-old woman in week 36 of pregnancy, had no

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Table 1. Case-patients with pandemic (H1N1) 2009, by age group, CEMIC University Hospital, Buenos Aires, Argentina, June 11–30, 2009

Case-patient age, y	No. (%) case-patients tested	Total case-patients*			Hospitalized case-patients		
		No. (%)†	95% CI‡	p value	No. (%)§	95% CI‡	p value
<5	63 (12)	37 (59)	45.6–71.0	0.019	19 (51)	34.4–68.0	0.352
5–18	44 (9)	19 (43)	28.3–59.0	0.631	3 (16))	3.4–39.6	0.250
19–59	338 (66)	194 (57)	51.9–62.7	0.003	47 (24)	18.4–30.9	0.304
≥60¶	68 (13)	25 (37)	25.4–49.3	—	9 (36)	17.9–57.4	—
Total	513 (100)	275 (54)	49.2–58.0	—	78 (28)	23.1–34.1	—

*Patients with subtype H1N1 infection confirmed by real-time reverse transcription–PCR.
†Percent of all patients tested.
‡CI, confidence interval.
§Percent of all patients with influenza virus subtype H1N1 infection.
¶Reference group.

underlying diseases and required mechanical ventilation within 24 hours of admission and for the next 33 days. During this period, 35 other case-patients with pandemic (H1N1) 2009 were hospitalized at CEMIC; of these 36 patients, 16 (44%) were admitted to the intensive care unit, and 10 (28%) required mechanical ventilation. The most common diagnosis for hospitalized patients was pneumonia; 42% had interstitial bilateral infiltrates, 31% had focal consolidation, and 4% had pleural effusion. Chest radiographs were unremarkable for 23% of hospitalized patients. The mean hospital stay was 7 days (range 2–42 days; median 9 days).

A total of 78 case-patients were hospitalized at CEMIC and other institutions in Buenos Aires. Six patients died, of whom 5 had an underlying condition: 3 were immunocompromised, 1 was obese and a chronic user of tobacco, and 1 had pulmonary and heart disease. Three patients were ≥60 years of age, 2 were in the 19–59-year age group, and 1 was an infant. Two hospitalized patients died at CEMIC. Both were ≥60 years of age, had underlying health conditions, and later developed bacterial sepsis; 1 also had bilateral pneumonia.

Underlying conditions were evaluated for all 275 patients with laboratory-confirmed influenza virus subtype H1N1 infection. For evaluation, hospitalized and ambulatory patients were divided into 2 age groups: <5 and ≥5 years of age. For patients ≥5 years of age, the presence of an underlying condition was significantly associated with a higher rate of hospitalization (p = 0.040). Of the 78 hospitalized patients, 56 (72%) had no underlying condition (Table 2).

Conclusions

From June 11–30, 2009, pandemic (H1N1) 2009 virus was detected in 54% of respiratory samples submitted to CEMIC's virology laboratory. This percentage is similar to the overall percentage reported by Argentina's National Reference Laboratory during the same period (*8*). However, initial reports from Mexico showed a lower positivity rate (29%, 2,582/8,817) (*9*). This difference may be partly caused by the different clinical criteria for respiratory specimen collection, site restrictions, or seasonal factors. The percentage of influenza subtype H1N1 cases detected during this study (54%) is apparently higher than that for seasonal influenza A strains (H1N1 and H3N2) detected at CEMIC University Hospital during the same epidemiologic weeks in previous years (19% and 26% in children and adults, respectively, as confirmed by immunofluorescence testing) (*10*) (M. Echavarría, unpub. data).

The high frequency of infection with pandemic (H1N1) 2009 virus likely resulted from the lack of population immunity to this antigenically novel H1N1 subtype. The lowest rate of infection was seen for patients ≥60 years of age. This finding may suggest that persons in this age group were previously exposed, through infection or vaccination, to an influenza A (H1N1) virus that

Table 2. Underlying clinical conditions in 275 case-patients with pandemic (H1N1) 2009, by age group, CEMIC University Hospital, Buenos Aires, Argentina, June 11–30, 2009

Clinical condition	Case-patients <5 y of age			Case-patients ≥5 y of age		
	No. (%) hospitalized	No. (%) outpatients	p value	No. (%) hospitalized	No. (%) outpatients	p value
None*	15 (79)	16 (89)	—	41 (69)	148 (83)	—
Underlying condition	4 (21)	2 (11)	0.659	18 (31)	31 (17)	0.040
Immunocompromised	1 (5)	1 (5.5)	0.999	9 (15)	11 (6)	0.028
Pregnant	0	0	—	1 (2)	4 (2)	0.999
Other†	3 (16)	1 (5.5)	0.603	8 (14)	16 (9)	0.205
Total	19	18	—	59	179	—

*Reference group.
†Hematologic malignancies, chronic lung disease (asthma, chronic obstructive pulmonary disease), chronic cardiac disease, diabetes and obesity.

is genetically and antigenically more closely related to pandemic (H1N1) 2009 than to other recent influenza A viruses (*11*).

Our finding that 66% of respiratory samples tested during this study were from patients 19–59 years of age is similar to findings in previous pandemic influenza situations. Furthermore, 49% of hospitalized patients in this study were 19–59 years of age. Most case-patients hospitalized with pandemic (H1N1) 2009 infection (72%) had no underlying medical condition, but a severe degree of lung involvement was observed in these patients: one third required mechanical ventilation support.

We report a mortality rate of 2% among the 275 confirmed pandemic (H1N1) 2009 case-patients followed in this study. A similar rate (2.3%) was reported by the National Ministry of Health during the same period (*8*). From May through December 4, 2009, a total of 11,234 cases were confirmed in Argentina, and 613 (5.5%) case-patients died (*5*). In the Americas, Argentina currently has the fourth highest number of deaths associated with influenza virus subtype H1N1, after the United States, Brazil, and Mexico (*12*). The reasons for these unusual epidemiologic features are the focus of ongoing investigations.

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Extensive Mammalian Ancestry of Pandemic (H1N1) 2009 Virus

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We demonstrate that the novel pandemic influenza (H1N1) viruses have human virus–like receptor specificity and can no longer replicate in aquatic waterfowl, their historic natural reservoir. The biological properties of these viruses are consistent with those of their phylogenetic progenitors, indicating longstanding adaptation to mammals.

In 2009, a new H1N1 influenza virus (pandemic [H1N1] 2009) emerged in Mexico, spread to the United States (*1*), and subsequently caused the first influenza pandemic of the 21st century (*2*). The emergence of pandemic (H1N1) 2009 virus is imperfectly understood, but an early switch in hemagglutinin (HA) receptor specificity is essential to allow interspecies transmission (*3*–*5*).

Pandemic (H1N1) 2009 virus strains were recently reported to be reassortants of the North American and European swine lineages (*6*). Phylogenetic evidence suggests that this reassortment event occurred 10–17 years ago (*7*). These data suggest that the current pandemic (H1N1) 2009 virus strains should have receptor specificity typically found in the HA of mammalian viruses (Neu5Acα2,6Gal). In addition, they may have lost the ability to replicate in avian hosts, the natural reservoir species. To test these hypotheses, we examined the biological properties of pandemic (H1N1) 2009 virus, including receptor specificity, erythrocyte binding, and ability to replicate in avian species.

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The Study

We first tested species-specific erythrocyte agglutination by the pandemic (H1N1) 2009 isolates A/California/04/2009 and A/Tennessee/1-560/2009 and by other isolates from humans, swine, and birds (Table 1). The pandemic (H1N1) 2009 isolates showed reduced or absent agglutination of goose and chicken erythrocytes. Human and swine H1N1 viruses were agglutinated by turkey, guinea pig, chicken, and goose erythrocytes, and all erythrocytes we tested except those of swine were agglutinated by avian isolates (Table 1).

We next measured the receptor binding of the 2 pandemic (H1N1) 2009 isolates to sialic substrates, both natural (fetuin) and synthetic (3′-sialyllactose [3′SL] and 6′-sialyllactosamine [6′SLN] attached to a polyacrylic carrier) (Figure). The binding pattern to fetuin was identical among all isolates tested (association constant $K_{ass} \approx 5.8 \pm 0.5$, 1/μM sialic acid). The currently circulating human and pandemic influenza (H1N1) viruses showed a preference for 6′SLN and negligible binding to the avian-type 3′SL. A similar pattern was observed for 2 recent swine viruses, which bound only to 6′SLN receptors with nearly equal affinity as pandemic (H1N1) 2009 isolates. As expected, the 2 avian H1 viruses bound strongly only to 3′SL (Figure).

To assess the infectivity and pathogenicity of pandemic (H1N1) 2009 virus strain A/California/04/2009 in terrestrial (chickens, quails) and aquatic (domestic and wild ducks) avian species, we inoculated 10 birds of each species by intranasal, intraocular, and intratracheal instillation with $\approx 10^{6.0}$ of the 50% egg infectious dose (EID_{50}) of the virus. We then observed the birds for the next 2 weeks for death and for viral shedding and signs of illness. No birds showed obvious clinical signs of disease. Virus was detected only on postinoculation day 1 in infected chickens and ducks and only in tracheal samples at low titers (≤ 1.7 $\log_{10}$ of the EID_{50}/mL [*8*]) (Table 2). However, no later shedding of virus was observed, indicating that the virus detected on postinoculation day 1 could have been caused by residual virus particles after inoculation. In contrast, our results revealed that the A/California/04/2009 strain efficiently infected quails with significantly higher titers (≤ 3.4 $\log_{10}$ EID_{50}/mL until postinoculation day 5; $p<0.05$) in both oropharyngeal and cloacal swab specimens (Table 2). The virus was detected in the trachea (1.7 $\log_{10}$ EID_{50}/g), lungs (2.3 $\log_{10}$ EID_{50}/g), and cecal tonsil (0.8 $\log_{10}$ EID_{50}/g) of quails on postinoculation day 5.

[1]These authors contributed equally to this article.

Table 1. Erythrocyte agglutination by representative human, pandemic, swine, and avian H1 influenza virus isolates

Virus isolate	Subtype	Hemagglutination titer of erythrocytes from indicated species, HAU*†					
		Turkey‡	Guinea pig‡	Chicken§	Goose§	Horse¶	Swine#
Human isolates							
A/Brisbane/59/2007	H1N1	64	64	64	64	<2	<2
A/New Jersey/15/2007	H1N1	32	32	16	16	<2	<2
Pandemic isolates							
A/California/04/2009	H1N1	64	64	4	16	<2	<2
A/Tennessee/1-560/2009	H1N1	32	32	<2	8	<2	<2
Swine isolates							
A/swine/North Carolina/007270/2008	H1N1	32	64	8	16	<2	<2
A/swine/Iowa/003479/2009	H1N1	64	64	32	32	2	<2
Avian isolates							
A/mallard/Alberta/66/2007	H1N4	64	64	32	32	16	<2
A/mallard/Alberta/496/2008	H1N4	64	64	32	32	16	<2

*HAU, hemagglutination units.
†Titers are expressed as the reciprocal of the highest virus dilution that yields complete HA agglutination.
‡Neu5Acα2,6Gal > Neu5Acα2,3Gal.
§Neu5Acα2,6Gal < Neu5Acα2,3Gal.
¶Neu5Gc2,3Gal.
#Neu5Gcα2,6Gal > Neu5Gcα2,3Gal.

The potential bird-to-bird intraspecies transmission of the A/California/04/2009 pandemic (H1N1) 2009 virus strain in avian species was also examined by introducing 3 contact birds to the inoculated birds' cages on postinoculation day 1. There was no subsequent evidence of viral shedding through the upper respiratory tract or fecal-oral route in any group of birds except 1 of 3 contact quails (Table 2). Oropharyngeal virus titers in this quail were l.7 and 1.5 $\log_{10}$ EID_{50}/mL on postinoculation days 3 and 5, indicating that productive viral replication was occurring.

Conclusions

The A/California/04/2009 pandemic (H1N1) 2009 virus strain showed minimal replication and no transmission in chickens and ducks (domestic and wild), but the virus replicated and had limited transmissibility in quails. Our finding is consistent with those of Swayne et al. (*9*). The inability of the virus to replicate efficiently in chickens and ducks could very well be linked to its human virus–like receptor recognition.

The ability of influenza A viruses to agglutinate erythrocytes from a variety of hosts may reflect the viruses' receptor specificity (*10*,*11*). We observed similar binding patterns for the mammalian influenza (H1N1) viruses, with the exception that the pandemic strains had reduced binding to chicken erythrocytes. This binding pattern was also observed with 1 of the swine isolates, suggesting it might be a trait of swine-adapted viruses. Taken together, a difference in the hemadsorption phenotype observed with erythrocytes from species with either less Neu5Acα2,6Gal or less Neu5Ac linkage overall could be explained by the mammalian origin of the novel pandemic (H1N1) 2009 influenza viruses.

To test this possibility, we measured the HA affinity of H1 influenza viruses from various species of origin for synthetic receptor analogues. All mammalian H1 viruses showed a typical human virus–like preference for the Neu5Acα2,6Gal-containing receptor 6′SLN. Compared with the currently circulating H1N1 human viruses, both pandemic (H1N1) 2009 strains and contemporary swine influenza virus (H1N1) strains were able to bind substantially more strongly (5–12×) to an α2,6-containing glycopolymer; the currently circulating subtype H1N1 human viruses are strictly adapted to this receptor (*12*). This feature demonstrated that pandemic H1N1 strains, which have a HA gene

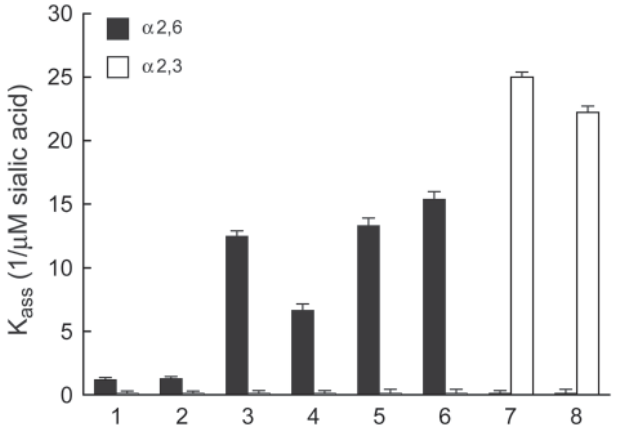

Figure. Receptor specificity of human, pandemic, swine, and avian H1 influenza viruses. Association constants (K_{ass}, 1/μM sialic acid) of virus complexes with sialylglycopolymers conjugated to 3′-sialyllactose (avian-like Neu5Acα2,3Gal-containing receptor, white bars) and 6′-sialyllactosamine (human-like Neu5Acα2,6Gal-containing receptor, black bars). Higher K_{ass} values indicate stronger binding. Values are the mean ± SD of 4 independent experiments (1/μM sialic acid). 1, A/Brisbane/59/2007; 2, A/New Jersey/15/2007; 3, A/California/04/2009; 4, A/Tennessee/1-560/2009; 5, A/swine/North Carolina/007270/2008; 6, A/swine/Iowa/003479/2009; 7, A/mallard/Alberta/66/2007; 8, A/mallard/Alberta/496/2008.

Table 2. Replication and transmission of influenza virus A/California/04/2009 (H1N1) in various bird species

Common name (genus and species)	Virus titer*						Transmission†
	1 dpi		3 dpi		5 dpi		
	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca	
Chicken (*Gallus gallus domesticus*)	1.7 ± 0.5	<	<	<	<	<	0
Domestic duck (*Anas platyrhynchos*)	1.7 ± 0.5	<	<	<	<	<	0
Wild duck (*Anas platyrhynchos*)	1.3 ± 0.0	<	<	<	<	<	0
Quail (*Coturnix japonica*)	3.4 ± 0.9‡	<	2.0 ± 1.3‡	0.8 ± 0.9	1.3 ± 0.9	1.3 ± 0.9	33‡

*dpi, days postinoculation; <, titer below limit of detection (<0.75 $\log_{10}EID_{50}$/mL). Virus titers were determined in eggs and are expressed as the $\log_{10}$ 50% egg infectious dose (EID_{50})/mL (*8*). Data are presented as means ± standard deviation of titers of positive samples (≥0.75 $\log_{10}$ EID_{50}/mL).
†Percentage of contact birds from which virus was isolated.
‡p<0.05, by 1-way analysis of variance.

of swine lineage, have retained their current swine virus–like binding characteristics despite their efficient spread in humans.

To identify substitutions in the HA molecule that could be responsible for the human-like receptor binding phenotype of the pandemic and contemporary swine influenza (H1N1) isolates, we compared the H1 HA sequences deposited in the Influenza Research Database (www.fludb.org). We observed that 99.99% of all swine viruses isolated after 1980 have Asp190 or Asn190. HA sequences of swine viruses isolated before 2000 harbor Gly225, whereas 92.3% of more recent classical swine viruses have Asp225. Crystallographic analysis of human and swine H1 HA has shown that Asp225 and Asp190 are responsible for human virus–like receptor specificity (*13*). Therefore, the human-like amino acids encoded at HA positions 190 and 225 in the novel pandemic and swine influenza (H1N1) viruses may at least partially explain their innate affinity for the human-type receptor.

Recent phylogenetic analysis showed that each segment of the pandemic (H1N1) 2009 virus is nested within a well-established swine influenza lineage for >10 years before the recent outbreak (*7*). Hence, the ancestors of this virus circulated undetected for about a decade before the virus emerged in humans. Our finding that contemporary swine viruses acquired the ability to recognize 6′SLN with at least 5-fold higher affinity than did human strains and completely lost the ability to bind to Neu5Acα2,3Gal provides clear evidence to support this hypothesis. It is possible that the progenitors of pandemic (H1N1) 2009 virus were accumulating enough mammal-associated changes to allow a refinement of their receptor-binding properties. Our findings substantiate that strong mammalian-like receptor specificity is a critical barrier to infection of various hosts with pandemic (H1N1) 2009 virus. Other biological factors associated with their adaptation and tissue tropism in humans will likely be identified in the future.

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Concurrent Silicosis and Pulmonary Mycosis at Death

Yulia Iossifova,[1] Rachel Bailey, John Wood, and Kathleen Kreiss

To examine risk for mycosis among persons with silicosis, we examined US mortality data for 1979–2004. Persons with silicosis were more likely to die with pulmonary mycosis than were those without pneumoconiosis or those with more common pneumoconioses. Health professionals should consider enhanced risk for mycosis for silica-exposed patients.

The pneumoconioses are a group of irreversible but preventable interstitial lung diseases, most commonly associated with inhalation of asbestos fibers, coal mine dust, or crystalline silica dust. In response to inquiries from silica-exposed workers concerned about diagnoses of coccidioidomycosis or cryptococcal meningitis for their co-workers, we examined whether excess risk for mycosis exists among persons with silicosis.

The Study

We examined mortality data from the National Center for Health Statistics of the Centers for Disease Control and Prevention, which are coded for causes of death reported on death certificates for all US residents according to the International Classification of Diseases (ICD) (www.cdc.gov/nchs/products/elec_prods/subject/mortmcd.htm). Because silicosis usually has a latency of ≥20 years, we restricted our analyses to persons ≥45 years of age at death. We separately evaluated 3 groups of persons who had died with pulmonary mycosis coded as either the underlying or the contributing cause of death (any mention on death certificate) during 1979–2004: 1) those with any mention of silicosis versus no mention; 2) those with any mention of asbestosis versus no mention; and 3) those with any mention of coal worker pneumoconiosis (CWP) versus no mention. We excluded decedents with ≥2 pneumoconioses.

We analyzed mortality data for persons assigned the following ICD codes (9th revision [ICD-9] 1979–1998; 10th revision [ICD-10] 1999–2004): 502/J62 (silicosis); 501/J61 (asbestosis); 500/J60 (CWP); and 110–118/B35–B49 (any mycosis). We defined pulmonary mycosis as death with ICD-9 and ICD-10 codes 112.4/B37.1 (candidiasis); 114/B38.0, B38.1, B38.2, B38.9 (coccidioidomycosis); 115/B39.0, B39.1, B39.2, B39.4, B39.9 (histoplasmosis); 116.0/B40.0, B40.1, B40.2, B40.9 (blastomycosis); 116.1/B41.0, B41.9 (paracoccidioidomycosis); 117.1/B42.0, B42.9 (sporotrichosis); 117.7/B46.0, B46.5, B46.9 (zygomycosis); 117.3/B44.0, B44.1, B44.9 (aspergillosis); 117.5/B45.0, B45.9 (cryptococcosis); and 118/B48.7 (opportunistic mycoses). For many mycoses, ICD-9 codes do not differentiate pulmonary from other types of mycoses. For ICD-10 codes, we limited data to mycoses coded as pulmonary, opportunistic, and some unspecified type of mycoses (e.g., B38.9, B39.4, B39.9, B40.9, B41.9, B42.9, B44.9, B45.9, B46.5, and B46.9). We provided results with and without ICD-9 code for opportunistic mycoses and ICD-10 codes for unspecified mycoses and opportunistic mycoses.

We computed prevalence rate ratios and 95% confidence intervals (CIs) to separately compare pulmonary mycosis prevalence at death among persons with silicosis, asbestosis, and CWP with that for persons in the referent group and to compare pulmonary mycosis prevalence at death among persons with silicosis with that for persons in the 2 pneumoconiosis comparison groups. Each ratio was computed by dividing the proportion of mycosis deaths in 1 group by the corresponding measure in the comparison group.

Decedents with pneumoconiosis and mycosis were rare, and most mycoses were pulmonary: 77% in persons with silicosis; 79% asbestosis, and 53% CWP (Table 1). Persons with silicosis were 4.5× (95% CI 3.4–6.0×) more likely to have any mycosis at death and 9.5× (95% CI 6.9–13.1×) more likely to have pulmonary mycosis at death than were those without pneumoconiosis. Persons with silicosis were 2.9× (95% CI 1.9–4.4×) more likely than those with asbestosis and 6.7× (95% CI 4.3–10.5×) more likely than those with CWP to have pulmonary mycosis at death.

Among persons who died with pneumoconiosis, aspergillosis was the most common pulmonary mycosis. Those with silicosis were more likely than those without any pneumoconiosis to have aspergillosis, coccidioidomycosis, or cryptococcosis at death (Table 2). Among the 48 decedents who had silicosis and mycosis, 9 also had tuberculosis, 4 had diabetes (2 with tuberculosis), 2 had lung malignancy (1 with tuberculosis), and none had received organ transplants. From 1987 (the first year of ICD codes for HIV) to 2004, 1 decedent with silicosis had HIV but not mycosis. Of 8 decedents with asbestosis and HIV, 2 had mycoses; no decedents with CWP had HIV. When we lim-

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Table 1. Cause of death with any death certificate mention of selected pneumoconioses and mycoses (underlying or contributing cause), US residents ≥45 y of age, 1979–2004*

		Mycoses, no. (%)				Prevalence rate ratio† (95% CI)	
		Any mention		Underlying cause		Any mention	
Cause	No. deaths	All	Pulmonary	All	Pulmonary	All	Pulmonary‡
Silicosis	6,723§	48 (0.71)	37 (0.55)	25 (52)	21 (57)	4.5 (3.4–6.0)	9.5 (6.9–13.1)
Asbestosis	23,899§	58 (0.24)	46 (0.19)	22 (38)	18 (39)	1.5 (1.2–2.0)	3.3 (2.5–4.4)
CWP	46,088§	72 (0.16)	38 (0.08)	29 (40)	16 (42)	1.0 (0.8–1.2)	1.4 (1.0–2.0)
All other deaths¶	51,677,216	81,699 (0.16)	29,914 (0.06)	33,941 (42)	12,982 (43)	NA	NA

*Data from National Center for Health Statistics (www.cdc.gov/nchs/products/elec_prods/subject/mortmcd.htm). CI, confidence interval; CWP, coal worker pneumoconiosis; NA, not applicable.
†Proportion of decedents with each selected pneumoconiosis who had mycosis coded as the underlying cause of death or as a contributing cause of death on the entity axis compared with the analogous proportion of pulmonary mycosis decedents without any mention of a selected pneumoconiosis as underlying cause of death or a contributing cause of death on the entity axis (all other deaths).
‡When International Classification of Diseases (ICD) 9th or 10th revision codes for opportunistic mycoses and ICD-10 codes for unspecified types of mycoses were excluded, the prevalence rate ratio and 95% CI for deaths with any mention of silicosis became 10.4 (7.5–14.4); asbestosis 3.3 (2.4–4.4); and CWP 1.5 (1.1–2.1).
§No. persons who died for which the selected pneumoconiosis was coded as the underlying cause of death or as a contributing cause of death on the entity axis, excluding decedents with multiple pneumoconioses.
¶Excludes deaths with any mention (underlying or contributing cause) of silicosis, asbestosis, and/or CWP.

ited analysis to decedents in the southwestern states (Arizona, California, Nevada, New Mexico, Texas, and Utah), those with silicosis were 27.9× (95% CI 12.6–62.0×) more likely to have coccidioidomycosis at death than were decedents without pneumoconioses, and those with asbestosis were 4.5× (95% CI 1.9–10.8×) more likely to have coccidioidomycosis at death than were decedents without pneumoconioses. Decedents with silicosis from states bordering the Ohio, Missouri, and Mississippi River valleys, were 4.8× (95% CI 0.7–34.0×) more likely to have histoplasmosis at death than were decedents without pneumoconiosis, and decedents with asbestosis were 3.9× (95% CI 1.3–12.2×) more likely to have histoplasmosis than were decedents without pneumonconioses.

Conclusions

We found that persons who die with silicosis are more likely to die with pulmonary mycosis than are those who die without pneumoconiosis or who die with the more common pneumoconioses. Insofar as silica dust impairs cellular defense, silica-exposed workers (without silicosis) may be at increased risk for fungal infections, as they are for mycobacterial infections (*1*).

Aspergillosis was the most common mycosis among persons with pneumoconiosis. Aspergillosis is a known complication in patients with underlying pulmonary disease, such as pulmonary tuberculosis and pneumoconiosis (*2*), in which silica-impaired macrophages are incapable of targeting inhaled conidia (*3*). The rarity of candidiasis in persons with silicosis may reflect the fact that healthy workers are less likely to have concurrent diabetes or HIV infection.

Concurrent mycosis was specific to decedents with silicosis compared with those with asbestosis or CWP, possibly because coal mine dust and asbestos fibers are less toxic to macrophages than are crystalline silica. Persons with asbestosis and CWP are also less commonly affected by autoimmune diseases and systemic immunologic complications than are those with silicosis (*4*,*5*).

Direct impairment of macrophage function by crystalline silica and poor drug penetration into silicotic lung nodules have resulted in high (>20%) treatment failure and relapse rates for patients with silicosis who are receiving chemotherapy for tuberculosis (*6*). This finding has prompted prolonged and more aggressive treatment of tuberculosis for such persons (*7*). Similarly, treatment of mycosis in

Table 2. Cause of death for decedents with any death certificate mention of selected pneumoconioses (underlying or contributing cause) and any mention of the most common types of pulmonary mycosis, US residents ≥45 y of age, 1979–2004*

	Pulmonary mycosis, prevalence rate ratio (95% CI)			
Cause	Coccidioidomycosis†	Histoplasmosis‡	Aspergillosis§	Cryptococcosis¶
Silicosis	17.9 (8.0–39.9)	2.9 (0.4–20.7)	13.9 (9.2 – 21.1)	6.9 (3.3 –14.5)
Asbestosis	4.2 (1.8–10.1)	3.3 (1.2–8.8)	5.5 (3.9– 7.8)	0.8 (0.3–2.6)
CWP	0	0.4 (0.1–3.0)	1.8 (1.2–2.9)	0.9 (0.4–1.9)

*Compared with persons who died without any mention of selected pneumoconioses. Data from National Center for Health Statistics (www.cdc.gov/nchs/products/elec_prods/subject/mortmcd.htm). CI, confidence interval; CWP, coal worker pneumoconiosis.
†Six with coccidiodomycosis among 6,723 decedents with silicosis; 5 among 23,899 decedents with asbestosis; zero among 46,088 with CWP; 2,576 among 51,677,216 without silicosis, asbestosis, and/or CWP.
‡One with histoplasmosis among 6,723 decedents with silicosis; 4 among 23,899 with asbestosis; 1 among 46,088 with CWP; 2,633 among 51,677,216 without silicosis, asbestosis, and/or CWP.
§Twenty-two with aspergillosis among 6,723 decedents with silicosis; 31 among 23,899 with asbestosis; 20 among 46,088 with CWP; 12,165 among 51,677,216 without silicosis, asbestosis, and/or CWP.
¶Seven with cryptococcosis among 6,723 decedents with silicosis; 3 among 23,899 with asbestosis; 6 among 46,088 with CWP; 7,786 among 51,677,216 without silicosis, asbestosis, and/or CWP.

patients with silicosis or substantial past exposure to silica dust may require prolonged treatment and possibly chronic suppressive antifungal therapy, as is used for patients with immunocompromised conditions (*8*).

Lacking population-based surveillance data for silicosis and mycosis illness and silica exposures, we relied on death certificate data, which have limitations; e.g., only ≈1 of 6 persons who had silicosis had silicosis recorded as a cause of death on the death certificate (*9*). Also, many persons with substantial exposure to silica dust never receive a diagnosis of silicosis. We were unable to address the question of possible increased risk for mycosis among silica-exposed persons, and our analysis may underrepresent the actual extent of concurrent silicosis and mycosis. Another limitation was use of ICD-9 and ICD-10 coding for fungal infections and for fungal infection causing death. Sensitivity of this method can vary at different institutions and over time, especially if fungal infections are underdiagnosed. In addition, the ICD-9 classification codes for many mycoses do not differentiate pulmonary from other types of mycoses.

Health professionals should consider enhanced risk for mycosis with regard to preventive interventions, differential diagnosis, and mycosis treatment of silica-exposed workers. Measures to protect silica-exposed workers with coexposure to fungi include reducing silica exposure; wetting soil and bird droppings to suppress fungal-contaminated dust; maintaining good personal hygiene; and, in areas with endemic inhaled fungi, using enclosed operator cabs with high-efficiency particulate air filtration or personal respiratory protection for particulates (*10*).

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Coccidioidomycosis among Scholarship Athletes and Other College Students, Arizona, USA[1]

Nicole G. Stern and John N. Galgiani

To compare coccidioidomycosis case rates among groups of young adults in a disease-endemic region, we reviewed medical charts for serologic testing and coding. Case rates were higher for scholarship athletes than for other students and paralleled 5× more serologic testing. Our findings underscore the need to routinely test patients for coccidioidomycosis.

Coccidioidomycosis, known commonly as valley fever, is a fungal infection endemic to the southwestern United States (*1*). The illness most commonly caused is community-acquired pneumonia (*2*,*3*). Some researchers estimate that ≈50,000 infections are severe enough to warrant medical attention (*1*,*4*); however, reported infections represent less than a quarter of this estimate, creating uncertainty about the true impact of valley fever.

Scholarship athletes receive financial support for their educations in return for participation in intercollegiate varsity competition. Athletes comprise a precisely defined group and afford a special opportunity to calculate endemic risk for infection within a young adult population. We calculated case rates for scholarship athletes and then compared the rates with those for other students and persons of college age.

The Study

The Campus Health Service at the University of Arizona is located on campus in Pima County. The average yearly enrollment during 1998–2006 of 36,000 was used for calculating case rates for all students (*5*). Scholarship athletes at the university total 475 annually.

Campus Health medical charts for January 1, 1998, through December 31, 2006, were reviewed for serologic testing for coccidioidomycosis. Charts were also reviewed for International Classification of Diseases, 9th edition (ICD-9), codes for coccidioidomycosis (114.0–114.5, 114.9) to identify students in whom the diagnosis was made by testing elsewhere. Athletes' charts were identified based on billing information.

Most serologic testing, identified by its current procedural terminology (CPT) code, was performed by the Campus Health laboratory, which used PREMIER *Coccidioides* enzyme immunoassay (Meridian Diagnostics, Cincinnati OH, USA). Occasionally, ≈10% of the time, serum samples were tested by a single commercial laboratory, which used immunodiffusion testing for precipitin-type or complement fixing–type antibodies (*6*). Any positive result was considered diagnostic. Students tested multiple times for coccidioidomycosis were counted once, either in the first year for which results were positive or in the year first tested if all results were negative. Individual charts were reviewed for those students who did not have coccidioidal testing done at Campus Health and were included only if the diagnosis could be corroborated by outside laboratory documentation. A case of coccidioidomycosis was defined as one in which the diagnosis could be corroborated by positive coccidioidal serologic results, fungal culture, or histologic identification of spherules in biopsy specimens.

In addition, charts of all athletes who had coccidioidal infection were reviewed for demographic information, type of sport (indoors vs. outdoors), and extent of disease. Arizona state statistics were provided by the Arizona Department of Health Services. Not all statistics for all age ranges were available. All data were analyzed by using SPSS 11.0 for Windows (SPSS Inc, Chicago, IL, USA). Significance tests used the Fisher exact χ^2. This study was approved by the University of Arizona Institutional Review Board and by the Campus Health Service Departmental Review Committee.

Of 2,754 student medical charts selected for review based on routine serologic testing for coccidioidomycosis or an ICD-9 code for coccidioidomycosis, 305 students were found to have coccidioidomycosis. Of these 305, a total of 297 had positive coccidioidomycosis results by serologic testing done at Campus Health and 8 were identified with diagnostic results from other laboratories. The resulting annual student case rate for all students was 94.1/100,000.

Annual rates for college students are 3–4× higher than available county and state rates for various age ranges (Table). The average age range of university students is 17–23 years. Because Pima County statistics includes cases reported to the state by Campus Health, actual differences between student rates and nonstudent Pima County rates for persons of similar ages would be even greater than represented here.

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Table. Annual case rates per 100,000 for coccidioidomycosis in University of Arizona students and in selected age groups in Pima County, Arizona, and Arizona, USA, 1998–2006*

Year	University of Arizona students	Age group, y: 15–24		15–19		20–24		25–29	
		Pima County	Arizona	Pima County	Arizona	Pima County	Arizona	Pima County	Arizona
1998	108								
1999	61								
2000	53	13.3	9.23						
2001	56	13.5	12.5						
2002	111	25.7	17.3						
2003	69	17.4	12.0						
2004	117	41	32.4	33.5	30.2	48.3	34.6	40.4	37.5
2005	147	53.2	30.1	39.7	23.6	66.3	36.7	64.9	38.4
2006	125	59.9	45.6	56.39	42.4	63.1	48.8	80.0	62.9

*The University of Arizona campus is located in Pima County. Blank cells indicate age ranges where composite statistics were not available.

Coccidioidal infections occurred in 16 scholarship athletes. The composite case rate, or annual incidence of coccidioidomycosis, was 374/100,000 (95% confidence interval [CI] 192–639) for scholarship athletes compared with 90/100,000 (95% CI 79–103) for other students ($p<0.00001$). In parallel, a much greater proportion (4.6%, 95% CI 3.9–5.4) of scholarship athletes were tested for coccidioidal antibodies than were other students (0.8%, 95% CI 0.77–0.84%) ($p<0.000001$). Infection for all but 1 athlete with disseminated infection was limited to the chest and/or skin, which resolved either untreated or after several months of antifungal therapy. The age distribution of scholarship athletes was the same as for other students, and only 1 of 16 athletes was from Arizona. Female athletes with coccidioidal infections comprised 56% of the total compared with 41% of all uninfected athletes ($p = 0.31$). For 81% of infected athletes, sports competition took place outdoors, whereas 88% of uninfected athletes competed outdoors ($p = 0.43$).

Conclusions

A recent study in Arizona found that <13% of patients with community acquired pneumonia were tested for coccidioidomycosis (*7*). In contrast, Campus Health Service at the University of Arizona tested ≈50% of students with community acquired pneumonia for coccidioidomycosis (*8*,*9*). Consequently, proportionately higher than age-adjusted Pima County case rates are found. Similarly, the case rate of 374/100,000 for scholarship athletes, >4× higher than that for other students, is associated with a >5-fold increase in coccidioidal testing. Therefore, it would seem that most, if not all, of the increased case-rate for athletes can be accounted for by increased testing; little, if any, increased susceptibility can be attributed to increased exercise or athletic training. We did not evaluate why clinicians tested more scholarship athletes. We speculate that etiologic diagnoses for any illness that reduced physical performance were sought more frequently for scholarship athletes than for other students. However, we cannot entirely exclude the possibility that the higher rate of testing was to some extent a consequence of higher rates of pneumonia in scholarship athletes. In any instance, it would appear that more complete testing for community acquired pneumonia associated with valley fever does result in considerably higher estimates of case rates for this fungal infection.

In the past, skin test conversion rates have suggested an annual infection rate of ≈3,000/100,000 (*10*). A 2007 telephone survey conducted by the Arizona Department of Health Services of persons reported with new diagnoses of coccidioidomycosis found that the median length of residence in an endemic area was 17 years (*11*), further suggesting an average annual infection rate of ≈3%. In this context, rates for scholarship athletes fall well within these other estimates.

Case rates among scholarship athletes were actually underrepresented in outdoor sports, and the spectrum of disease severity was in line with that found in past studies of the student population as a whole (*8*). Many scholarship athletes come from regions where coccidioidomycosis is not endemic. Because fewer of these students had prior coccidioidal infection, the annual risk for the group as a whole would be slightly increased as shown in a previous report from the same college health group (*9*). Arizona state statistics show a marked increase with age in case rates, e.g., 163 cases/100,000 for persons ≥65 years of age. Our findings do not discount the possibility that immune responses of elderly patients differs from those of younger patients; disease in the elderly is more severe (*12*). However, our study suggests that at least an equal proportion of young adults have clinical illness from coccidioidal infection sufficient to seek medical evaluation.

Our findings address infection among college students, particularly scholarship athletes, and support a recommendation that student health clinicians and team physicians consider coccidioidomycosis as a possible etiology of illness in students who live in or have traveled recently to disease-endemic regions. More generally, our findings reinforce practice guidelines revised by the Infectious

Diseases Society of America for management of community-acquired pneumonia to assist clinicians in evaluating patients with endemic exposure (*13*).

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Novel Human Bocavirus in Children with Acute Respiratory Tract Infection

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Human bocavirus (HBoV) and HBoV2, two human bocavirus species, were found in 18 and 10 of 235 nasopharyngeal aspirates, respectively, from children hospitalized with acute respiratory tract infection. Our results suggest that, like HBoV, HBoV2 is distributed worldwide and may be associated with respiratory and enteric diseases.

Acute respiratory tract infection (ARTI) is a leading cause of illness and death in infants and young children (*1*). Human bocavirus (HBoV) was first identified in respiratory samples from children and was proposed to be pathogenic in humans (*2*). Subsequently, HBoV infections were reported in children with ARTI (*1*,*3*,*4*), and found in stool samples from children with gastroenteritis worldwide (*5*). In 2009, Kapoor et al. identified another human parvovirus that was most closely related to HBoV and named it HBoV genotype 2 (HBoV2) (*6*). In this study we examined its presence in nasopharyngeal aspirates (NPAs) from children hospitalized with ARTI.

The Study

NPA samples were collected from 235 children hospitalized with ARTI at the First Hospital of Lanzhou University, Gansu Province, China during December 2007–November 2008. All patients were ≤15 years of age, and informed consent was obtained from their parents. Demographic data and clinical findings were recorded. The study protocol was approved by the hospital ethics committee.

DNA and RNA were extracted from the NPAs by using QIAamp DNA and QIAamp viral RNA mini kits (QIAGEN, Beijing, China). The cDNA sample was synthesized by using random hexamer primers. A standard reverse transcription–PCR was used to screen for human rhinovirus, respiratory synctial virus (RSV), influenza virus A, influenza virus B, parainfluenza virus 1–3, human metapneumovirus, human coronavirus (HCoV)-NL63, and HCoV-HKU1, and PCR method was used to screen for adenovirus (ADV) (*7*–*11*).To screen for HBoV, PCR was performed by using primers 188F and 542R, as described by Allander et al. (*2*). For HBoV2, nested PCR was performed with sf1/sr1 and sf2/sr2 primers, which amplified a 455-bp fragment of the partial NS1 gene, as described (*6*).

In addition, we designed HBoV2 forward (SN1: 5′-ACCAGTGGGAGAACCACAAG-3′) and reverse (SN2: 5′-GGCATTTGTTTCCATGCTTT-3′) primers, which produced a 563-bp fragment of the NP1 gene of HBoV2. Positive and negative controls were included for each PCR. Purified PCR products were sequenced by using SinoGenoMax. ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) was used to align the obtained sequences with sequences available in GenBank.

In total, 260 viruses were identified in 196 (83.4%) of the 235 children. Using nested PCR, we found 21 positive specimens; further nucleotide sequence analysis showed that 10 (4.3%) were HBoV2 and 11 were HBoV (Figure, panel A). All 11 HBoV strains detected by using HBoV2 nested-PCR were included in the 18 HBoV-positive patients as determined by PCR using primers 188F and 542R. Of the 10 HBoV2-positive patients, 7 (70%) were co-infected with other respiratory viruses, including 4 patients with RSV. Of the 18 HBoV-positive patients, 12 (66.7%) displayed co-infections. There were no statistically significant differences in the HBoV2 and HBoV detection ($p = 0.119$ by χ^2 test) and co-infection ($p = 1.000$ by Fisher exact test) rates.

Of the 10 HBoV2-positive patients, 9 were male and 1 was female ($\chi^2 = 1.957$, $p = 0.162$). The median age was 8.5 months, and 9/10 (90%) were ≤3 years old. HBoV2 infections were detected throughout the year. Of the 18 patients who were HBoV positive, 11 were male and 7 were female ($\chi^2 = 0.084$, $p = 0.772$). The median age of patients was 11.5 months, and 16/18 (88.9%) were ≤3 years old.

HBoV infections were detected in every month except August, with peaks in December (3 cases) and January (4 cases). The main diagnoses of the 3 patients with HBoV2 monoinfection were acute asthmatic bronchopneumonia, bronchopneumonia, and acute upper respiratory tract infection in 1 patient each. For the 6 patients with HBoV monoinfection, the main diagnoses were acute asthmatic bronchopneumonia (4 cases) and bronchopneumonia (2 cases). The clinical signs and symptoms of HBoV2 and HBoV positive patients included cough, fever, sputum production, crack-

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les, wheezing, rhinorrhea, cyanosis, vomiting, and diarrhea (Table 1). For patients with HBoV2 monoinfection, the median hospital stay was 11.3 days (range 4–23 days), and 2 had underlying illnesses (idiopathic pulmonary hemosiderosis and iron deficiency anemia). The chest radiograph of 1 patient showed upper middle zone air-space shadows.

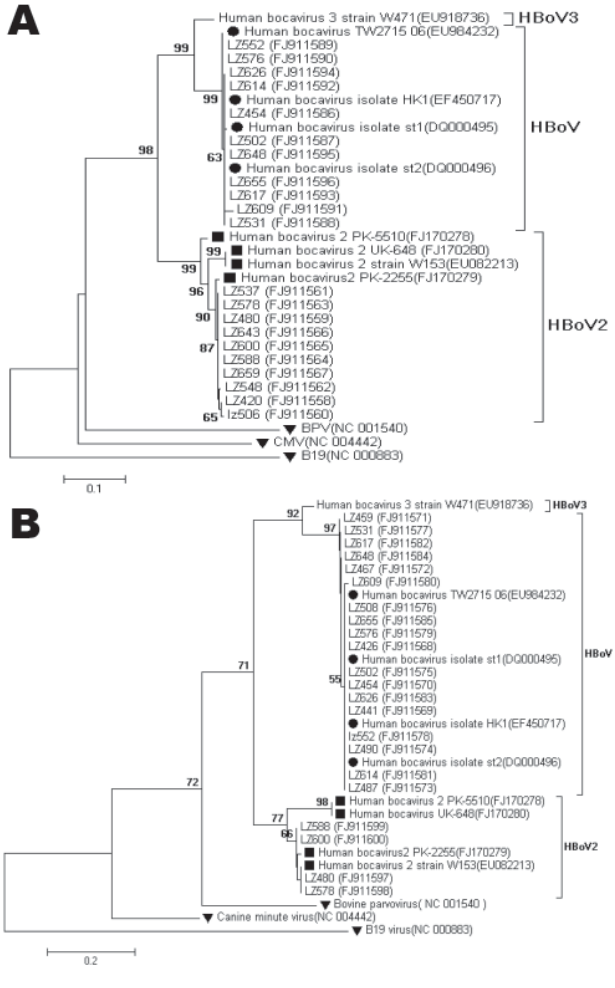

Figure. Phylogenetic analysis of A) the partial nonstructural protein 1 (NS1) nucleotide sequences (412 bp) and B) the partial nucleoprotein 1 (NP1) nucleotide sequences (256 bp) of human bocavirus 2 (HBoV2), Gansu Province, People's Republic of China. The phylogenetic trees were constructed by the neighbor-joining method using MEGA 3.1 (www.megasoftware.net), and bootstrap values were determined for 1,000 replicates. Bootstrap values >50% are shown at the branching points. Human bocavirus (HBoV) and HBoV2 reference sequences are indicated by circles and squares, respectively. Bovine parvovirus (BPV), cytomegalovirus (CMV), and human parvovirus B19 (B19) reference sequences are indicated by inverted triangles. Scale bars indicate nucleotide substitutions per site. Reference sequences were obtained from GenBank (accession nos. DQ000495, DQ000496, EF450717, EU082213, EU918736, EU984232, FJ170278, FJ170279, FJ170280, NC001540, NC004442, and NC000883). Sequences generated in this study were deposited in GenBank under accession nos. FJ911558–FJ911600.

For patients with HBoV monoinfection, the median hospital stay was 7.8 days (range 6–10 days), and none had underlying illnesses. Chest radiographs of 2 patients showed shadows in the left lung zone.

Ten HBoV2 NS-1 sequences (455 bp) shared 98%–99% and 95%–96% nucleotide sequence identity and 99%–100% and 98%–99% deduced amino acid sequence identity with HBoV2 strain PK-2255 (FJ170279) and HBoV2 strain W153 (EU082213), respectively; These sequences also shared 81%–82% and 83.3%–84.4% nucleotide sequence identity and 90% and 88% deduced amino acid sequence identity with the HBoV prototype strain ST1 or ST2 (DQ000495 and DQ000496) and human bocavirus 3 strain W471 (EU918736), respectively. The 4 HBoV2 NP-1 sequences shared 98%–99% and 97.6%–98.3% nucleotide sequence identity and 98%–100% and 97%–100% deduced amino acid sequence identity with HBoV2 strain PK-2255 and HBoV2 strain W153, respectively, and shared 74%–78% and 69.7%–70.3% nucleotide sequence identity and 69%–73% and 58%–62% deduced amino acid sequence identity with the prototype strain ST1 or ST2 and human bocavirus 3 strain W471, respectively. The nucleotide and deduced amino acid sequences of NS-1 and NP-1 shared high identities (>97%) with the HBoV2 and HBoV sequences (Table 2). Phylogenetic analysis indicated that HBoV2 is more closely related to HBoV (Figure).

Conclusions

Using nested PCR and sequencing, we identified HBoV2 infections in 10 (4.3%) of 235 NPAs from children hospitalized with ARTI. Most of the patients were ≤3 years old. In HBoV2-positive patients, co-infection was high (70%), with RSV being the most common co-pathogen. Primers SN1 and SN2 were designed to detect the NP1 gene in the 10 HBoV2-positive patients. However, only 4 gave positive results, which occurred because of the low PCR sensitivity with this pair of primers and because the NP1 gene of HBoV2 is divergent, as described (*6*). Furthermore, as previous studies (*6*,*12*) pointed out, potential recombination upstream from the NP1 gene may explain the lower detection. Phylogenetic analysis showed that the NS-1 region of the HBoV2 strain (LZ480 and LZ578) clustered closely with that of the HBoV2 PK-2255 strain (FJ170279), and the NP-1 region clustered closely with that of the HBoV2 W153 strain (EU082213), suggesting potential recombination in the HBoV2 strains (Figure). In addition, 11 HBoV sequences were amplified by using nested-PCR for HBoV2. In the future, HBoV2-specific primers should be designed to investigate the prevalence of HBoV2 and its potential association with disease.

We found no difference in the clinical symptoms or length of hospital stay between the groups with HBoV2 and HBoV monoinfection, as well as between the groups with

Table 1. Comparison of clinical characteristics among different groups of children with acute respiratory infections, Gansu Province, China*

Characteristic	Group 1, n = 3	Group 2, n = 7	Group 3, n = 6	Group 4, n = 12	p values		
					Group 1 vs. Group 2	Group 3 vs. Group 4	Group 1 vs. Group 3
Male sex	3 (100)	6 (85.7)	3 (50)	8 (66.7)	0.499†	0.145†	1.000†
Age ≤3 y	3 (100)	6 (85.7)	6 (100)	10 (83.3)	1.000†	0.307†	1.000†
Median duration of hospitalization, d (range)	11.3 (4–23)	9 (8–11)	7.8 (6–10)	10.3 (5–20)	0.492†	0.187‡	1.000‡
No. underlying diseases	2	1	0	1	0.183†		
Clinical diagnosis							
AURI	1 (33.3)	0	0	1 (8.3)			
Suppurative tonsillitis	0	0	0	1(8.3)			
Acute asthmatic bronchopneumonia	1 (33.3)	4 (57.1)	4 (66.7)	7 (58.3)	0.500§	0.572§	0.405§
Bronchopneumonia	1 (33.3)	1 (14.3)	2 (33.3)	3 (25.0)	0.533§	0.561§	0.774§
Acute bronchitis	0	2 (28.6)	0	0			
Clinical signs							
Fever	2 (66.7)	5 (71.4)	2 (33.3)	7 (58.3)	0.708§	0.310§	0.405§
Cough	2 (66.7)	5 (71.4)	5 (83.3)	10 (83.3)	0.708§	0.730§	0.583§
Wheeze	1 (33.3)	4 (57.1)	4 (66.7)	7 (58.3)	1.000§	0.572§	0.405§
Rhinorrhea	2 (66.7)	2 (28.6)	2 (33.3)	5 (41.7)	0.500§	0.572§	0.405§
Sputum production	1 (33.3)	5 (71.4)	5 (83.3)	4 (33.3)	0.500§	0.066§	0.226§
Crackles	2 (66.7)	6 (85.7)	6 (100)	7 (58.3)	1.000§		
Vomiting	0	1 (14.3)	1 (16.7)	0			
Diarrhea	2 (66.7)	1 (14.3)	1 (16.7)	1 (8.3)	0.183§	0.569§	0.226§

*Values are no. (%) children except as indicated. HBoV, human bocavirus; AURI, acute upper respiratory tract infection. Group 1, HBoV2 monoinfection; Group 2, HBoV2 co-infection; Group 3, HBoV1 monoinfection; Group 4, HBoV1 co-infection.
†By χ^2 test.
‡By Mann-Whitney test.
§By Fisher exact test.

HBoV2 monoinfection and HBoV2 co-infection (Table 1). Statistical analysis indicated that HBoV2 and HBoV co-infection obviously did not correlate with disease severity (data not shown). Two of 3 patients with HBoV2 monoinfection had diarrhea with no vomiting (Table 1), and only 1 of 10 patients who were HBoV2 positive vomited. Further investigation is needed to exclude oral or inhaled gastric viruses as possible sources of NPA-associated HBoV2. Phylogenetic analysis showed a high degree of similarity between HBoV2 sequences found in China and those in other areas (Figure). Our results suggest that like HBoV, HBoV2 is distributed worldwide and may be associated with respiratory and enteric diseases. Additional studies are needed to confirm the association between human bocavirus species (HBoV2 and HBoV) and respiratory tract infections or other diseases.

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Table 2. Nucleotide and amino acid sequence comparisons among and between human bocaviruses, Gansu Province, China*

Virus comparison	NS1 sequence identity, %		NP1 sequence identity, %	
	Nucleotide	Amino acid	Nucleotide	Amino acid
With HBoV2 PK-2255 sequence	98–99	99–100	98–99	98–100
With HBoV2 strain W153 sequence	95–96	98–99	97.6–98.3	97–100
With the HBoV prototype strain ST1 or ST2 sequence	81–82	90	74–78	69–73
With HBoV 3 strain W471 sequence	83.3–84.4	88	69.7–70.3	58–62
Among the HBoV2 sequence	97–99	98	98–100	99–100
Among the HBoV sequence	97–98	99	97–100	98–100

*HBoV, human bocavirus; NS1, nonstructural protein 1; NP1, nucleoprotein 1.

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Lymphocytic Choriomeningitis Virus Meningitis, New York, NY, USA, 2009

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We describe a case of lymphocytic choriomeningitis virus (LCMV) meningitis in a New York, NY, resident who had no apparent risk factors. Clues leading to the diagnosis included aseptic meningitis during winter and the finding of hypoglycorrachia and lymphocytosis in the cerebrospinal fluid. LCMV continues to be an underdiagnosed zoonotic disease.

Lymphocytic choriomeningitis virus (LCMV) belongs to the family *Arenavirus* and has been found in Europe, the Americas, Australia, and Japan. Arenaviruses are typically associated with rodents, which can become chronically infected. The viruses are zoonotic and transmissible to man. LCMV is primarily maintained in the common house mouse (*Mus musculus* and *M. domesticus*), but has also been reported among pet and research rodents, including hamsters and guinea pigs (*1*). The prevalence of LCMV in wild mice in the United States ranges between 3.9% and 13.4% (*2*). Human cases demonstrate a fall-to-winter predominance corresponding to the movement of rodents indoors (*1*). Limited data are available on human infections but serologic studies in Washington, DC, Baltimore, Maryland, and Birmingham, Alabama, identified evidence of previous infections among 2%–10% of the population sampled (*3*). A study in a Washington, DC, military base found that 8%–11% of patients having a febrile neurologic illness between 1941 and 1958 were seropositive for LCMV(*4*)

Transmission occurs by inhalation (aerosol and droplet), fomites, or direct contact with excreta or blood from infected rodents. Human-to-human transmission has been reported during pregnancy from infected mothers to the fetus (*5*) and through solid organ transplantation (*6*). The incubation period is 1–2 weeks. Infections in persons with an intact immune system are often asymptomatic or result in a mild self-limiting illness including fever, chills, myalgia, and headache. Photophobia, anorexia, testicular or parotid pain, pharyngitis, and cough have also been noted (*1*). Leukopenia, thrombocytopenia, and mild liver function abnormalities are common and may last 1–3 weeks. Central nervous system invasion is seen only in a few patients either after an initial febrile illness or, less commonly without any early symptoms. During this neurologic phase, most patients have aseptic meningitis and a peripheral leukocytosis (*1*). Cerebrospinal fluid (CSF) leukocyte cell counts often exceed 1,000/μL, and glucose levels are low. Infection is rarely fatal, although ascending paralysis, transverse myelitis, or encephalitis may develop (*1*).

Critical or even fatal outcomes from LCMV are usually associated with transplacental infections, and, more recently, solid organ transplantation. Congenital LCMV can result in hydrocephalus, chorioretinopathy, macrocephalopathy, or microcephalopathy and can mimic the classic TORCH (toxoplasmosis/*Toxoplasma gondii*, other infections, rubella, cytomegalovirus, herpes simplex virus) pathogens. In 1 study of 26 infants with congenital LCMV, 9/26 (35%) died and 10/16 (63%) survivors had severe neurologic sequelae (*7*). Transplacental infection presumably occurs after maternal viremia during the first and second trimesters (*8*). In 2003 and 2005, the first reports of infection after solid organ transplantation were reported in which 7 of 8 patients receiving LCMV-infected organs (kidney, liver, and lung) died (*6*). Therapy for LCMV is supportive, although limited data support the use of ribavirin in immunosuppressed patients. We report a case of meningitis caused by LCMV in New York City.

The Patient

A 49-year-old man (taxi driver) sought treatment during the winter of 2009. He had a 7-day history of fever, chills, headache, nausea, vomiting, neck rigidity, generalized weakness, and paresthesias in his fingers. He denied recent travel or exposure to sick contacts. Medical history included a corneal transplant the previous year. His temperature when admitted was 37.5°C. Physical examination identified nuchal rigidity but was otherwise unremarkable. CSF showed leukocytes 871 cells/μL; erythrocytes 2 cells/μL, with 93% lymphocytes and 7% monocytes; glucose 32 mg/dL (serum glucose 93 mg/dL); and protein 185 mg/dL. Results of a computerized axial tomography scan of the brain and chest radiograph were normal. A complete blood count and results of serum chemical tests and liver function tests were unremarkable. Blood cultures yielded no growth. CSF cultures for bacterial, fungal, and mycobacterial organisms were negative. Results for syphilis

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rapid plasma reagin, cryptococcal antigen, and HIV testing were negative.

Because of the patient's hypoglycorrachia, pronounced CSF leukocytosis, and negative CSF cultures, acute infection with LCMV was considered. Serum tested at a commercial laboratory was positive for LCMV (immunoglobulin [Ig] G titer >256 (reference <16) and IgM titer 80 (reference <20) by immunofluorescence assay. CSF collected on day 1 and serum collected on days 7 and 15 was tested for LCM antibodies by the Centers for Disease Control and Prevention's Special Pathogens Branch using ELISA. CSF and serum were both IgM and IgG positive and a 4-fold rise in acute-phase and convalescent-phase serum titers indicated the patient had a recent infection with LCMV (Table). The patient improved with supportive care, recovered fully, and was discharged on day 12.

Table. CSF and serum LCMV ELISA titers for specimens obtained from a male patient, New York, NY, USA, 2009*

Hospitalization day	Specimen type	LCMV IgM ELISA	LCMV IgG ELISA
1	CSF	1,280	QNS
7	Serum	6,400	100
15	Serum	6,400	400

*CSF, cerebrospinal fluid; LCMV, lymphocytic choriomeningitis virus; Ig, immunoglobulin; QNS, quantity not sufficient. Titers <100 in serum and <25 in CSF are considered negative.

Conclusions

The incidence rate of LCMV infection in New York City is unknown. The disease is likely underdiagnosed because only a limited number of commercial laboratories offer LCMV testing. Diagnosed cases of LCMV are often identified because they are part of a larger outbreak. Since 1960, only 7 cases have been reported in the literature (*8*,*9*); all but 2 had had contact with rodents. Several large outbreaks have been reported, often associated with pet hamsters or laboratory mice or hamsters; 1 outbreak had >236 human cases (*8*). A total of 54 cases of congenital LCMV are cited in the literature, 34 since 1993 (*5*).

Our patient denied both hamster and mouse exposure. His corneal transplant had occurred >1 year before, and was unlikely to be the source of infection. A definitive diagnosis was difficult, but testing by Centers for Disease Control and Prevention confirmed the diagnosis.

New York City Health Department officials attempted an investigation of the patient's home and surroundings to look for evidence of rodents or rodent excreta, but were unable to do so due to the patient's noncompliance. Several clues have assisted clinicians in the identification of patients with acute LCMV infection: 1) aseptic meningitis or encephalitis during the fall–winter season, 2) a febrile illness followed by brief remission before onset of neurologic illness, and 3) CSF with a lymphocytosis and hypoglycorrachia. Patients with suspected LCMV should be asked about potential rodent exposure (*1*). Patients with a history of organ transplantation within the preceding 3 months should be fully evaluated to determine whether infected organs were the source of LCMV.

We recommend enzyme immunoassay-based testing of CSF and serum when LCMV is considered. Public health surveillance, rodent control, healthcare provider education, and improved laboratory testing can enhance recognition of illness.

Health departments should consider making LCMV a reportable condition, and, if aseptic meningitis is reportable, questions should be added to case investigation forms regarding rodent exposure. LCMV was made reportable in New York City in 2009. Only with a better understanding of the true incidence of LCMV will authorities be able to enact measures to better prevent and control this disease in the vulnerable sections of our society.

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Severe Leptospirosis in Hospitalized Patients, Guadeloupe

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We evaluated prognostic factors for leptospirosis in 168 consecutive hospitalized patients in Guadeloupe. Factors independently associated with severity included chronic hypertension or chronic alcoholism, late initiation of antibacterial therapy, abnormal chest auscultation results, icterus, oligoanuria, disorders of consciousness, elevated aspartate aminotransferase levels, hyperamylasemia, and *Leptospira interrogans* serovar Icterohemorrhagiae.

Leptospirosis is a reemerging infectious disease in tropical and subtropical regions (*1*). In Guadeloupe, it has long been a major public health concern. Its incidence rate was ≈5.5/100,000 inhabitants per year from 1991 through 2002. Since 2003, this rate has greatly increased, peaking at 41.2/100,000 inhabitants in 2004 (*2*,*3*). The clinical features of leptospirosis vary and may progress to multiorgan failure and death (*4*). Initial clinical symptoms and laboratory test results associated with severe forms remain unclear. In this study, we focused on severe forms and determined prognostic factors that may help physicians in the early management of leptospirosis. We also characterized reservoir hosts by identifying the serovars of infecting strains. These findings will help establish appropriate control and prevention measures.

The Study

This study was conducted in the hospital of Pointe-à-Pitre (1,100 beds), a tertiary referral center for Guadeloupe and neighboring islands. The ethical committee of the hospital approved the study. All consecutive patients hospitalized from January 2003 and through December 2004 with confirmed leptospirosis were included; patients admitted to Guadeloupe's other hospital (200 beds) were excluded. Leptospirosis was confirmed if 1 blood culture yielded *Lesptospira* spp. or if specific antibodies were detected with either a single titer of ≥400 with the microscopic agglutination test (MAT) and an in-house enzyme immunoassay (EIA) with an immunoglobulin (Ig) M titer ≥400 (*5*) or at least a 4-fold increase in the MAT titer between the acute and convalescent phases. Cases were considered severe if dialysis (in case of oliguria) or mechanical ventilation was required or if the patient died. *Leptospira* serovars were isolated and identified as previously described (*3*). Epidemiologic, clinical, and laboratory data were collected retrospectively from medical records taken at patient's admission. Data were analyzed by using Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA). A multiple stepwise logistic regression analysis (SPSS, Chicago, IL, USA) was performed for variables with a p value ≤0.2.

During the 2-year period, leptospirosis was diagnosed in 168 hospitalized patients. A total of 132 case-patients had specific antibodies (49 had a single MAT titer ≥400 and EIA IgM titer ≥400, and 83 showed a 4-fold increase in the MAT titer in paired serum samples); 36 cases were confirmed only by culture. Of the 132 case-patients testing positive for antibody, 19 were also positive for bacterial culture. All but 2 case-patients were residents of Guadeloupe; the other 2 were a tourist from Paris and a resident of the Dominica. The ethnic distribution of the study population was similar to that of the Guadeloupean population.

We assessed patients' demographic, epidemiologic, and clinical characteristics (Table 1). Twenty-four (14%) cases were considered to be severe: 6 (25%) of these were fatal. Female case-patients were significantly older than male case-patients (mean 58.5 ± 17.9 and 47 ± 15.9 years, respectively; p = 0.01). Chronic alcoholism was common (39%), especially among the 6 case-patients who died (67%). Chronic hypertension was also frequent (32%) (Table 1). The most common symptoms were myalgia (95%), headache (77%), digestive disorders (63%), fever (57%), abdominal pain (52%), and icterus (49%). Alveolar infiltrates was the most common feature, accounting for 9 (41%) of 22 anomalies observed in the lung by chest radiograph, followed by interstitial pattern (27%) and pleural suffusion (18%). Six case-patients with severe disease had cardiac complications: 2 had pericarditis confirmed by echocardiography, 2 had ischemic cardiac shock, and 2 myocardiopathy and myocarditis. Tomodensitometry or ultrasonography showed acute pancreatitis in 10 case-patients, of whom 6 had chronic alcoholism and 3 had severe disease. Thrombocytopenia (<150 × 10^9 cells/L) was common (90% of case-patients), with severe thrombocytopenia (<50 × 10^9 cells/L) observed in 19% of case-patients (Table 2).

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Hepatic cytolysis (alanine aminotransferase level >119 U/L or aspartate aminotransferase level >102 U/L) was found in 45% of case-patients. One fifth of case-patients exhibited rhabdomyolysis with creatinine phosphokinase levels >1,000 U/L (Table 2). The *L. interrogans* serovar Icterohemorrhagiae was found in 18 (45%) of the 40 case-patients for whom serovars were identified. The closely related *L. borgpetersenii* serovars Arborea and Castellonis accounted for 35% of identified strains (Table 2).

Univariate analysis showed that, after stratification for sex, severity was associated with age for women but not for men. Neither occupation (farming, livestock farming, construction, and gardening) nor contact with swine, cattle, or rodents was linked to severity (Table 1). Nine host-related factors (listed in order of decreasing odds ratio) remained independently associated with severity in the multivariate analysis: history of chronic hypertension, hyperamylasemia, history of chronic alcoholism, abnormalities at chest aus-

Table 1. Demographic, epidemiologic, and clinical characteristics as a function of severity among 168 case-patients with confirmed leptospirosis, Hospital of Pointe-à-Pitre, Guadeloupe, French West Indies, 2003–2004*

	Univariate analysis†				Multivariate analysis	
Characteristics	All case-patients, N = 168	Case-patients with severe disease, n = 24	Case-patients with nonsevere disease, n = 144	p value	OR (95% CI)	p value
Male sex	143/168 (85.1)	18/24 (75)	125/144 (86.8)	0.2	2.6 (0.4–17.7)	0.3
Age, y, mean ± SD (no. patients)						
F	58.5 ± 17.9 (25)	55.5 ± 2.4 (6)	36.7 ± 8 (19)	**0.05**		
M	47 ± 15.9 (143)	51.8 ± 6.5 (18)	49.6 ± 2.9 (125)	0.5		
Exposure to occupational risk‡	74/133 (55.6)	12/20 (60)	62 /113 (54.8)	0.6		
Contact with swine	41/115 (35.6)	5/10 (50)	36/105 (34.2)	0.5		
Contact with cattle	36/113 (31.9)	1/10 (10)	35/103 (34)	0.8		
Contact with rodents	56/114 (49.1)	3/9 (33.3)	53/105 (50.5)	0.5		
Medical history						
Duration of illness before antibacterial therapy§ >10 d	21/141 (14.9)	7/22 (31.8)	14/119 (11.8)	**0.01**	4.8 (1.1–20.2)	**0.032**
Diabetes mellitus	13/75 (17.3)	3/22 (13.6)	10/53 (18.9)	0.5		
Chronic hypertension ¶	24/75 (32)	9/22 (40.9)	15/53 (28.3)	0.2	30.9 (6.0–157.4)	**<0.001**
Chronic alcoholism#	29/75 (38.7)	11/22 (50.0)	18/53 (34)	0.2	16.8 (4.1–57.9)	**<0.001**
Initial features						
Hypothermia (<36.5°C)	28/144 (19.4)	7/23 (30.4)	21/121 (17.3)	0.1	4.6 (0.9–24.6)	0.07
Hyperthermia (>37.7°C)	82/144 (56.9)	10/23 (43.5)	72/121 (59.5)	0.1	3.8 (0.7–21.2)	0.12
Hypotension, SBP <100 mm Hg	24/143 (16.8)	7/20 (35)	17/123 (13.8)	**0.02**	0.3 (0.1–1.8)	0.2
Myalgia	73/77 (94.8)	12/13 (92.3)	61/64 (95.3)	0.6		
Consciousness disorders	10/119 (8.4)	4/13 (30.8)	6/106 (5.7)	**0.01**	3.8 (1.1–13.2)	**0.035**
Nuchal rigidity	10/116 (8.6)	2/12 (16.7)	8/104 (7.7)	0.2		
Headache	70/91 (76.9)	5/7 (71.4)	65/84 (77.4)	0.7		
Conjunctival suffusion	46/116 (39.6)	4/12 (33.3)	42/104 (40.4)	0.4		
Icterus	57/117 (48.7)	9/12 (75)	48/105 (45.7)	0.1	5.9 (1.1–31.1)	**0.036**
Hemorrhage**	15/165 (9.1)	5/24 (20.8)	10/141 (7.1)	**0.04**	4.2 (0.3–67.9)	0.31
Hepatosplenomegaly	30/86 (34.9)	7/11 (63.6)	23/75 (30.7)	**0.02**	1.7 (0.2–13.9)	0.62
Abdominal pain	64/124 (51.6)	14/18 (77.8)	50/106 (47.2)	**0.009**	3 (0.7–13.2)	0.139
Digestive disorders (diarrhea, vomiting)	66/105 (62.8)	12/15 (80)	54/90 (60)	0.2	3.5 (0.7–18.0)	0.12
Abnormalities at chest auscultation††	19/135 (14.1)	8/17 (47)	11/118 (9.3)	**<0.001**	8.7 (1.8–41.3)	**0.006**
Chest radiologic anomalies‡‡	22/112 (19.6)	6/16 (37.5)	16/96 (16.7)	**0.05**	0.8 (0.1–10.4)	0.85
Alveolar infiltrate	9/112 (8.0)	5/16 (31.2)	4/96 (4.2)	**<0.001**		
Electrocardiographic disorders§§	22/73 (30.1)	5/16 (31.2)	17/57 (29.8)	0.9		
Oliguria¶¶ or anuria	34/128 (26.6)	10/23 (43.5)	24/105 (22.9)	**0.04**	5.6 (1.5–20.6)	**0.009**

*OR, odds ratio; CI, confidence interval; SBP, systolic blood pressure. **Boldface** indicates significance.
†All values no. case-patients/no. examined (%) except as indicated.
‡Farming, livestock farming, construction, and gardening.
§Ampicillin or cefotaxime.
¶As reported by patients with specific therapy.
#Defined as alcohol dependence.
**Hemoptysis, hematuria, purpura, bleeding of the gums, and hematemesis.
††Crackles or ronchi.
‡‡Only anomalies of the lungs.
§§Excluding patients >60 y of age or with sinus tachycardia.
¶¶Urinary volume <400 mL/d.

Table 2. Laboratory findings for 168 case-patients with confirmed leptospirosis, Hospital of Pointe-à-Pitre, Guadeloupe, French West Indies, 2003–2004*

	Univariate analysis				Multivariate analysis	
Laboratory findings	All case-patients, N = 168†	Case-patients with severe disease, n = 24†	Case-patients with nonsevere disease, n = 144†	p value	OR (95% CI)	p value
Prothrombin time <70%	10/118 (8.5)	5/21 (23.8)	5/97 (5.1)	**0.01**	0.7 (0.05–9.8)	0.077
Thrombocytopenia, <50 × 10^9 cells/L	25/135 (18.5)	8/23 (34.8)	17/112 (15.2)	**0.02**	1 (0.1–2.4)	0.96
Hyperneutrophilia, >12 × 10^9 cells/L	24/136 (17.6)	10/23 (43.5)	14/113 (12.4)	**0.01**	0.9 (0.1–7.1)	0.93
ALT >119 U/L	30/108 (27.8)	6/23 (26.1)	24/85 (28.2)	0.8		
AST >102 U/L	58/128 (45.3)	17/23 (73.9)	41/105 (39)	**0.02**	4.3 (1.2–14.6)	**0.021**
CPK >1000 U/L	22/108 (20.4)	5/18 (27.8)	17/90 (18.9)	0.4		
LDH >800 U/L	11/101 (10.9)	3/17 (17.6)	8/84 (9.5)	0.3		
Amylase >285 U/L	16/82 (19.5)	8/15 (53.3)	8/67 (11.9)	**<0.001**	18.5 (3.8–88.8)	**<0.001**
Lipase >60 U/L	7/40 (17.5)	2/8 (25)	5/32 (15.6)	0.8		
Hemoglobin, g/dL	12.9 ± 0.6 (136)	11.1 ± 2.0 (23)	13.2 ± 3.4 (113)	**0.01**		
Hemoglobin <10 g/dL	13/136 (9.5)	5/23 (21.7)	8/113 (7.1)	**0.04**	2.3 (0.5–9.8)	0.2
Bilirubin, mg/dL	8.3 ± 2.3 (86)	15.2 ± 11.7 (18)	5.8 ± 7.2 (68)	**<0.001**		
Bilirubin >7 mg/dL	31/86 (36.0)	13/18 (72.2)	18/68 (26.4)	**<0.001**	0.48 (0.05–4.7)	0.6
Potassium, mmol/L	4.0 ± 0.22 (136)	3.8±.9 (23)	4.0 ± 1.3 (113)	0.5		
Sodium, mmol/L	133.5 ± 4.2 (136)	132.8 ± 5.3 (23)	133.6 ± 3.9 (113)	0.5		
Creatinine, mg/d	2.0 ± 0.3 (130)	2.8 ± 2.5 (21)	1.9 ± 2.0 (109)	**0.05**	2.0 (0.6–6.6)	0.2
Creatinine >1.5 mg/dL	54/130 (41.5)	11/21 (52.4)	43/109 (39.4)	0.2		
Urea nitrogen mg/dL	69.0 ± 10.2 (136)	85.8 ± 76.2 (23)	64.2 ± 64.2 (113)	0.5		
Isolation of *Leptospira* in blood culture	55/88 (62.5)	8/14 (57.1)	47/74 (63.5)	0.3		
L. interrogans serovar Icterohemorrhagiae	18/40‡ (45)	6/8 (75)	12/32 (37.5)	0.06	5.3 (1.0–26.0)	**0.004**
L. borgpetersenii serovar Castellonis	5/40‡ (12.5)	0/8	5/32 (15.6)	0.1		
L. borgpetersenii serovar Arborea	9/40‡ (22.5)	2/8 (25)	7/32 (21.9)	0.6		
L. kirschneri serovar Bogvere	6/40‡ (15)	0/8	6/32 (18.7)	0.1		
L. santarosai serovar Tabaquite	2/40‡ (5)	0/8	2/8 (25)	0.4		

*OR, odds ratio; CI, confidence interval; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatinine phosphokinase; LDH, lactate dehydrogenase. **Boldface** indicates significance.
†Mean ± SD (examined) or no. case-patients/no. examined (%).
‡Serovar identification was completed for 40 of the 55 *Leptospira* strains isolated.

cultation, oligoanuria, late initiation (>10 days after onset of symptoms) of antibacterial therapy, elevated aspartate aminotransferase levels, consciousness disorders, and icterus. Chronic alcoholism was linked to death ($p<0.01$). The *L. interrogans* serovar Icterohemorrhagiae was isolated in 75% of severe cases, but in only 38% of nonsevere cases, and was independently associated with severity.

Conclusions

The potential correlation between disease severity and *Leptospira* serovar remains a matter of debate. The *L. interrogans* serovars Icterohemorrhagiae, Canicola, and Australis have been linked to severity and multiorgan failure (*6*–*8*), but other studies did not confirm any link between serovar and outcome (*9*–*11*). *L.* Icterohemorrhagiae was clearly linked to disease severity. Therefore, a diagnostic test specifically detecting this serovar at an early stage of disease could help in the management of leptospirosis in patients in Guadeloupe. We confirmed that the *L. borgpetersenii* serovars Arborea and Castellonis, rarely isolated elsewhere, are highly prevalent in Guadeloupe (*3*,*12*). Taken together, they are the second most prevalent serovars after Icterohemorrhagiae. Notably, the serogroup Ballum, comprising the serovars Arborea and Castellonis, also is one of the main serogroups associated with human infections in Barbados (*9*). The serovar Arborea has been associated with mice, particularly in Barbados (*1*,*13*). In Guadeloupe, this serovar has been isolated from mice and rats, and the serovars Icterohemorrhagiae and Bogvere have been isolated from rats (*3*; V. Michelle, pers. comm.). Thus, rodent populations may be the main source of *Leptospira* spp. in Guadeloupe. Further animal studies are needed to establish the nature of these *Leptospira* reservoirs.

Chronic hypertension has not previously been found to predict poor prognosis for leptospirosis. Here, we found it to be the strongest risk factor for severe disease. Whether patients with histories of chronic hypertension are especially susceptible remains to be confirmed.

In our series of patients, acute hepatitis and pancreatitis were severe complications of leptospirosis in those with chronic alcoholism; chronic alcoholism itself was an independent indicator of poor prognosis. These results are consistent with findings from other studies conducted in La Réunion, another French overseas territory, and in continental France (*14*,*15*).

Patients with chronic hypertension or chronic alcoholism, late initiation of antibacterial therapy, consciousness disorders, abnormal features at chest auscultation, oligoanuria, jaundice, hyperamylasemia, or high aspartate aminotransferase levels may benefit from early intensive and specific management. The predominance of the Icterohemorrhagiae serovar, linked to severe disease, and of the Arborea and the Castellonis serovars highlights the need for rodent control to reduce the effects of leptospirosis in Guadeloupe.

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Seropositivity for *Enterocytozoon bieneusi*, Czech Republic

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To determine seropositivity for *Enterocytozoon bieneusi* in the Czech Republic, we tested 115 serum samples from various groups. We found that 20% from HIV-positive persons, 33% from persons with occupational exposure to animals, and 10% from healthy persons were positive by indirect immunofluorescence assay. Proteins of 32 kDa were detected in serum samples from seropositive persons.

Microsporidia are small, single-celled, obligate intracellular parasites that were initially characterized as eukaryotic protozoa, but they have recently been reclassified as fungi. Since 1985, microsporidia have been identified as a cause of opportunistic infections associated with persistent diarrhea and weight loss in persons with AIDS (*1*). Because of heightened awareness and improved diagnostic methods, microsporidia infections have been recognized in a wide range of human populations, including organ transplant recipients, travelers, children, contact lens wearers, the elderly, and immunocompetent persons with no known risk factors (*2*).

Of the 14 species of microsporidia known to infect humans, *Enterocytozoon bieneusi* is the most common and is associated with diarrhea and systemic disease (*3*). Symptomatic *E. bieneusi* infections are primarily found in immunodeficient persons, although infection in immunocompetent populations is increasingly detected (*4*). It is unclear whether asymptomatic microsporidia infections persist in immunocompetent persons and can reactivate during conditions of immune compromise and are than able to be transmitted to others at risk, such as during pregnancy or through organ donation.

Studies focusing on risk factors associated with microsporidiosis will help define more clearly the sources of microsporidia that pose a risk for transmission in the environment so that preventive strategies can be implemented. To determine seropositivity for *E. bieneusi* in the Czech Republic, we used 2 serologic assays for detecting *E. bieneusi*–specific antibodies in serum specimens from HIV-positive and HIV-negative persons and from blood donors and persons with occupational exposure to animals.

The Study

The National Institute of Public Health in Prague provided anonymous serum samples, originally collected for HIV diagnostics in 2007, from HIV-positive persons (n = 70) and healthy blood donors (n = 30). In addition, serum specimens from persons who worked with animals and animal excrement were collected after informed consent was obtained in 2007 (n = 15). Every specimen in the study was supplemented with data on the patient's clinical symptoms (e.g., indigestion, abdominalgia). The study was approved by the Hospital České Budějovice, a.s. ethics committee (protocol no. 202/07). The serum specimens were frozen directly after recovery and were stored at –20°C. Patient identifiers were removed from the samples before testing.

E. bieneusi spores were purified from positive stool samples, originally obtained from an HIV/AIDS patient from Lima, Peru (provided by G.S. Visvesvara, Centers for Disease Control and Prevention, Atlanta, GA, USA), by using Percoll and cesium chloride gradient centrifugation as previously described (*5*). The spore suspension was stored in phosphate-buffered saline (PBS) supplemented with antimicrobial drugs at 4°C. The purity of spore suspension was tested by using light microscopy (optical brightener staining), and the background reactivity of serum specimens with bacteria was observed by using indirect immunofluorescence antibody (IFA) assay.

IFA was performed with purified whole *E. bieneusi* spores at a concentration of 10^5/well. The serum samples were diluted in PBS by serial dilution, 1:10, 1:50, 1:100, 1:200, and 1:400, and results were compared with negative (1:100) and positive (1:400) control serum specimens. Serum specimens with titers ≥100 were considered positive on the basis of positive control serum titration. A total of 115 human serum samples were examined by IFA for antimicrosporidial immunoglobulin G. Specific antibodies against *E. bieneusi* were detected for 22 persons (19%; 95% confidence interval [CI] 12%–28%); 20% of HIV-positive persons (CI 11%–31%), 10% of blood donors (CI 2%–26%), and 33% of persons with animal risk exposure were positive (CI 11%–61%). CIs were calculated by the Clopper-Pearson formula for binominal counts (Table). None of the persons had demonstrated any clinical symptoms (e.g., loose stool, indigestion). The titers were higher (400) for HIV-positive persons and 1 animal keeper; the highest titer in blood donors was 200. No background reac-

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Table. Seroprevalence of *Enterocytozoon bieneusi* in different groups, Czech Republic

Serum source	No. positive/no. examined (%)	95% confidence interval, %*	Maximum titer
HIV-positive persons	14/70 (20)	11–31	400
Blood donors	3/30 (10)	2–26	200
Persons with animal exposure	5/15 (33)	11–61	400
Total	22/115 (19)	12–28	400

*95% Clopper-Pearson confidence interval for binomial counts.

tivity was observed in tested serum samples with bacteria present in spore suspension.

Proteins from 10^{10} purified spores were obtained by disruption of spores by using FastPrep 120 homogenizer and FastProtein Blue kit (both BIO-101, Inc., MP Biomedicals, Irvine, CA, USA) according to manufacturer's instructions. Proteins were separated by using preparative 4%–20% acrylamide gradient Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA, USA), electrotransferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience, Inc., Keene, NH, USA), and cut into strips. Each strip was incubated with a 1:100 dilution of individual serum specimens in 0.3% Tween-PBS, peroxidase-conjugated goat antibody (Bio Source International Inc., Camarillo, CA, USA) diluted 1:4,000 in 0.05% Tween-PBS, and blots were developed by diaminobenzidine substrate solution containing H_2O_2. Although several different proteins were identified in specimens from seropositive persons, a parasite protein with a molecular weight of ≈32 kDa was predominant, this protein was not identified in any of the negative serum specimens (Figure). The results of immunoblot testing correlated with those of IFA; all serum samples with a titer >200 showed a strong reaction with immunodominant antigen in immunoblot.

Conclusions

The epidemiology of human *E. bieneusi* infection is poorly understood, and environmental factors that affect transmission of the organism have not been fully elucidated. Most reports addressing prevalence of microporidiosis are based on coprologic or PCR diagnostics, and the serologic screening of humans for microsporidia infection has mostly been limited to species that can be cultured in vitro (*6*–*8*).

Our survey was performed on a limited sample size from highly selected populations, which resulted in decreased statistical power. Although our findings are likely minimal estimates, given the uncertain duration of serologic response and <100% sensitivity of testing, they showed a 33% seroprevalence of *E. bieneusi* among animal keepers and 20% among HIV-positive persons. In studies in which infection was diagnosed by detection of *E. bieneusi* spores or DNA in stool, infection rates ranged between 1.4% and 78% (*9*–*12*). However, PCR and coprology are not able to discriminate *E. bieneusi* spores that have simply been consumed and passed through the intestinal tract from those resulting from active infection. In contrast, the detection of specific antibodies indicates that these persons experienced infection.

In the healthy population represented by normal blood donors, we detected a prevalence of only 10%, which is similar to previously reported prevalences (1.3%–8.0%) of *Encephalitozoon*-specific antibodies among HIV-negative persons such as blood donors, slaughterhouse workers, dog breeders, forestry workers, and pregnant women (*6*–*8*). In other studies, microsporidia infection of immunocompetent travelers with self-limiting diarrhea has been reported (*13*). The persistence of microsporidia despite resolution of the intestinal disorder suggests microsporidia infection may cause clinical signs (e.g., diarrhea) during the early stages of infection that resolve even though the microsporidia persist. In our study, the highest seroprevalence was in the group with professional exposures (33%), concurrent with a high titer of specific antibodies. Some of these professionals cared for pigs on farms, where *E. bieneusi* spores have been found in the feces of up to 94% of pigs

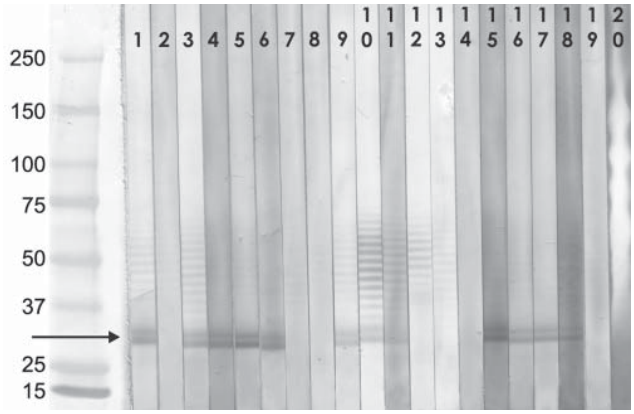

Figure. Western blot analysis of serum reactivity to *Enterocytozoon bieneusi* proteins, Czech Republic. Serum selection: HIV-positive persons (indirect fluorescence antibody [IFA] assay titers ≥400); blood donors, professionals with risk exposure (IFA titers ≥200). Serum samples diluted 1:100. Molecular weight markers (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA): lane 1, positive control (HIV/AIDS patient with proved *E. bieneusi* infection); lane 2, negative control (seronegative blood donor); lanes 3–8, selected samples from HIV-positive persons (3–6 IFA positive); lanes 9–14, selected samples from blood donors (9–11 IFA positive); lanes 15–20, selected samples from persons with occupational exposure to animals (15–18 IFA positive). Arrow indicates the 32-KDa protein fraction. Values on the left are in kilodaltons.

(*14*). Other studies also confirm the possibility of occupational risk exposure to microsporidia spores. An immunocompetent laboratory worker occupationally exposed to *Encephalitozoon cuniculi* remained seropositive 38 months after treatment (*15*). These results indicate the possible role of animals as a zoonotic source of microsporidia spores and show a possible occupational risk for persons who work with animals and animal excrement.

Studies that focus on risk factors associated with microsporidiosis will more clearly define the environmental sources of microsporidia that pose a risk for transmission so that preventative strategies can be implemented. Because no data exist about latent infection in immunocompetent carriers, possible infection reactivation and person-to-person transmission risk through organ donation, our future studies will focus on detailed seroprevalence data among healthy populations, especially persons with occupational risk exposure, and will aim to elucidate the role of various animals in human infection. This information may lead to better identification of possible sources of microsporidial infections and help effect their prevention.

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Hendra Virus Outbreak with Novel Clinical Features, Australia

Hume Field, Kylie Schaaf, Nina Kung, Craig Simon, David Waltisbuhl, Heather Hobert, Frederick Moore, Deborah Middleton, Allison Crook, Greg Smith, Peter Daniels, Ron Glanville, and David Lovell

To determine the epidemiologic and clinical features of a 2008 outbreak of Hendra virus infection in a veterinary clinic in Australia, we investigated the equine case-series. Four of 5 infected horses died, as did 1 of 2 infected staff members. Clinical manifestation in horses was predominantly neurologic. Preclinical transmission appears likely.

Hendra virus (HeV) was first identified in 1994 after a fatal respiratory disease occurred in horses and humans in Australia (*1*,*2*). Fruit bats (*Pteropus* spp.) are the natural reservoir (*3*). Eleven bat-to-horse spillovers have now been identified, with 4 resulting in horse-to-human transmission. Infection in horses typically has been characterized by acute febrile illness with rapid, progressive respiratory system compromise and high case-fatality rates (*2*,*4*–*8*). A high case-fatality rate was again a feature of the 2008 outbreak, which we describe here: 4 of 5 infected horses died, as did 1 of 2 infected veterinary clinic staff. In contrast to previously reported cases, the clinical features of the infected animals and humans reflected primarily central nervous system involvement. We describe the equine case-series and discuss the epidemiologic features.

The outbreak occurred at an equine referral veterinary practice in Brisbane, Australia. Thirty-seven horses were residing on the premises (Figure 1). Five confirmed cases (all in horses admitted for unrelated illnesses) occurred from June 26 through July 24, 2008 (online Appendix Table, www.cdc.gov/EID/content/16/2/338-appT.htm).

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The Equine Case-Patients

Case-Patient 1

Case-patient 1, a thoroughbred gelding 11 years of age, had resided at the clinic since June 27, 2007. On June 16, 2008, he was moved from yard GK to yard 19. On the morning of June 26, he showed ataxia, depression, disorientation, and extreme hypersensitivity when approached; the manifestations progressed to head tilting, left-side facial nerve paralysis, and endotoxemia. His condition deteriorated over several hours and he was euthanized (Table). A retrospective diagnosis of HeV infection was based on results of immunohistochemical testing on formalin-fixed cerebral tissue.

Case-Patient 2

Case-patient 2, a thoroughbred gelding 2 years of age, was admitted on June 17 with bullous keratopathy of the right eye. He was placed in stall 2. On the afternoon of June 30, he was depressed, inappetant, and ataxic and had an elevated rectal temperature. Over the ensuing days, he exhibited a right-sided head tilt, facial nerve paralysis, unsteadiness, circling to the right, and intermittent recumbency. On July 4, he was asymptomatic. A July 7 blood sample yielded a titer of 2,048 in an HeV neutralization test. A blood sample obtained on July 10 yielded a titer of

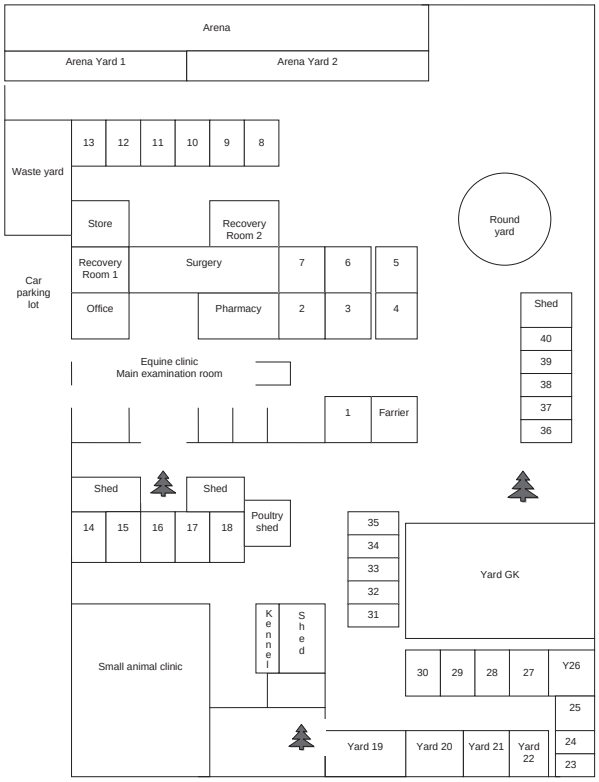

Figure 1. Layout of veterinary clinic where outbreak of Hendra virus infection occurred in horses, Australia, 2008. Individual horse stalls and yards are numbered 1–40. All yards are open, with yards 19–22 having a roofed shelter within.

Table. Clinical laboratory findings for confirmed equine cases of Hendra virus infection, Australia, 2008*

Case-patient no.	Hematologic/biochemical	Gross necropsy findings
1	Marked polycythemia, leukopenia, hyperkalemia, and hypochloremia; elevated levels of creatinine, bilirubin, globulins, and creatinine phosphokinase	Xanthochromic CSF
2†	Elevated bilirubin	
3	Elevated levels of globulins, creatinine phosphokinase, and fibrinogen; decreased bicarbonate	Xanthochromic CSF
4	NT	NT
5	NT	Unremarkable

*CSF, cerebrospinal fluid; NT, not tested.
†Case-patient 2 recovered but was euthanized.

4,096 and a positive real-time PCR result. He was moved to stall 24 on July 18. Serologic monitoring until he was euthanized on August 15 showed high titers by HeV neutralization test. A range of tissue specimens (liver, spleen, kidney, meninges, brain, spinal cord, and multiple lymph nodes), collected at necropsy, were positive by PCR and immunohistochemical testing.

Case-Patient 3

Case-patient 3, a quarter horse gelding 5 years of age, was admitted on June 12 with conidiobolus nasal granulomas and placed in stall 40. Subsequently, he was moved to stall 16 (June 16–19), stall 17 (June 20–July 3), yard 26 (July 4), and stall 3 (July 5). On the evening of July 4, he was pyrexic and subdued. On July 5, he showed severe depression, ataxia, disorientation, and stranguria, and his condition progressed to recumbency. He was euthanized on the afternoon of July 5, and a necropsy was performed (Table). HeV was diagnosed on the basis of positive PCR results from nasal swabs and blood samples. Multiple tissues collected at necropsy were subsequently found to be positive by immunohistochemical testing. On the basis of the length of the known incubation period of HeV infection, we believe that case-patient 3 was likely housed in stall 17 at the time of exposure to infection.

Case-Patient 4

Case-patient 4, a stock horse stallion 10 years of age, was admitted on May 15 with a fractured mandible. During June 17–24, he was in stall 1, and from June 25, in stall 4. On the morning of July 7, he was pyrexic, ataxic, severely depressed, disoriented, stranguric, and intermittently recumbent. Blood and urine specimens were positive for HeV by PCR. He was euthanized on July 8, and a limited necropsy was performed (Table). Lung tissue was positive by PCR, and subsequently, by virus isolation. Case-patient 4 was most likely housed in stall 4 at the time of exposure to infection.

Case-Patient 5

Case-patient 5, a stock horse mare 4 years of age, was admitted on June 24 with cutaneous tumors. She was placed in stall 5 until July 8, recovery room 2 until July 11, and then in stall 3. She exhibited a fever on July 23 and on July 24 was depressed, intermittently recumbent, and periodically pawing, head pressing, and leaning against the wall. A variety of tissues (meninges, brain, heart, and intestine) collected at necropsy were positive for HeV by PCR. The virus was subsequently isolated from kidney, spinal cord, spleen, and lymph node samples.

Conclusions

The flagrant neurologic features in this outbreak strongly contrast with those of previous cases, in which acute respiratory disease predominated. However, mild neurologic signs were noted in several horses in 1994 (*4*), and nonrespiratory manifestations (colic, depression) also have been observed previously (*7*). Phylogenetic analyses indicate some genetic differences between the strain that caused this outbreak and previously identified strains (K. Halpin, pers. comm.), but experimental infection of horses with the outbreak strain produced both respiratory and neurologic signs (D. Middleton, unpub. data). Virus dose and route of infection could plausibly influence clinical features; alternatively, the neurologic predominance in this outbreak may simply indicate the spectrum of possible manifestations.

Patient records and epidemiologic evidence support the fact that case-patient 1 had the primary case. A roost of *Pteropus alecto* and *P. poliocephalus* bats, recognized reservoirs of Hendra virus (*9*), was located within 5 km of the practice, and bats were regularly observed in the immediate practice vicinity. The reported incubation period for HeV infection in horses is 4–16 days (*1*,*4*). We contend that case-patient 1 was exposed to infectious body fluids from a foraging bat (through contamination of pasture, feed, water,

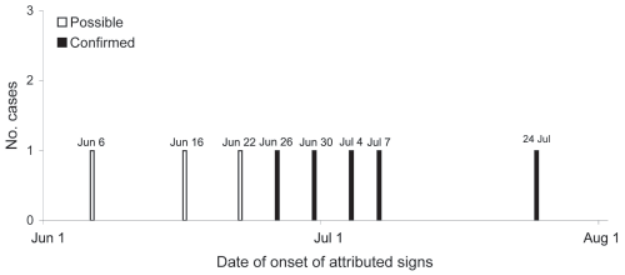

Figure 2. Epidemic curve of Hendra virus infection in horses, Australia, 2008. White bars represent the 3 possible cases; black bars represent the 5 confirmed cases.

or yard rails) and that he was the originator of this outbreak. In the 4 days before his illness and euthanasia, case-patient 1 had been treated at the clinic for a minor laceration. The clinic was the operational hub of the veterinary practice and the evident focus of transmission in this outbreak. We believe that transmission to case-patients 2–4 followed contamination of surfaces or equipment by infectious body fluids (plausibly blood, urine, saliva, or nasal discharge) from case-patient 1 before onset of his clinical signs.

Experimental studies suggest that horses may be infectious 48 hours before they show clinical signs (D. Middleton, unpub. data) At the time, case-patients 2–4 were housed in the clinic area or were being intensively treated, with daily or twice daily visits to the clinic, which would likely have increased their probability of becoming infected. Environmental swab samples from stalls 2, 3, and 4 yielded positive results by PCR, confirming that surfaces (walls, doors, wire mesh partitions between stalls) had been contaminated. Case-patient 5 had 3 possible sources of infection: most plausibly, case-patient 4, who had been in the adjacent stall until July 8; less plausibly, the recovered case-patient 2, who had been in stall 2 until July 18; and least plausibly, environmental contamination in stall 3 (occupied by case-patient 3 until July 5). The horse in yard 20, who had an extended opportunity for direct contact with case-patient 1, and the horses in stalls 6 and 7, who had extended opportunity for close contact with case-patients 2 and 5, respectively, did not acquire infection. These findings support previous observations that unassisted horse-to-horse transmission is inefficient. Three other horses died at the clinic without a definitive diagnosis before the death of case-patient 1, but the lack of clinical samples, conflicting epidemiologic evidence, and plausible alternative reasons for death precluded their inclusion as case-patients. However, they are considered possible case-patients (Figure 2).

Results of PCR and serologic tests for all other horses on the premises remained negative 4 weeks after case 5 occurred. No further cases have occurred.

Veterinarians and horse owners should consider HeV infection in any horse exhibiting acute-onset febrile illness, regardless of clinical manifestations, and implement appropriate risk assessment and management strategies. Biosecurity Queensland has compiled comprehensive veterinary guidelines (*10*)

Addendum

Two additional spillover events have since occurred: in June 2009, with 3 horses and 1 human fatally infected, and in August 2009, with at least 1 horse fatally infected and several reported human exposures. The outcome of the latter are unknown at the time of writing. Two additional spillover events have since occurred: in June 2009, 3 horses and 1 human were fatally infected (with a fourth horse nonfatally infected); and in August 2009, two horses were fatally infected.

Acknowledgments

We acknowledge and sincerely thank our colleagues at Biosecurity Queensland, Queensland Health, and the Commonwealth Scientific and Industrial Research Organisation Australian Animal Health Laboratories for their dedication and expertise in the field and laboratory during the outbreak response.

This article is dedicated to our friend and colleague, Ben Cunneen, who lost his life to Hendra virus in this outbreak.

Dr Field is a veterinary epidemiologist with Animal Biosecurity, Queensland Primary Industries and Fisheries, Brisbane He played a key role in the identification of fruit bats as the natural hosts of Hendra virus in Australia and Nipah virus in Malaysia and was part of the team that identified bats as the reservoir for a cluster of severe acute respiratory syndrome–like coronaviruses.

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The Critical Role of Permanent Voucher Specimens of Hosts and Vectors in Public Health and Epidemiology

A. Townsend Peterson

Zoonotic disease transmission systems constitute sets of interacting species, ranging from pathogens in wildlife reservoirs and transmitted directly to humans (*1*), pathogens in wildlife reservoirs and transmitted to humans by vectors (*2*), to pathogens in complex systems of multiple interacting definitive hosts, intermediate hosts, and vectors (*3*). Because these systems are so tightly linked to biodiversity, studies must include sampling of diverse species. Emerging Infectious Diseases journal is replete with such studies documenting the circumstances surrounding a disease case or outbreak. However, in developing these studies, researchers have been remiss regarding 1 major element of documentation of their work. Other elements are documented carefully. For example, when sequence data are extracted, primer sequences are presented in the Materials and Methods section, and numbers are given of sequences deposited in the GenBank database. Biodiversity samples, in contrast, are often identified cursorily or incompletely, and the documentation (i.e., the host or vector animal from which the pathogens were isolated) is discarded. I suggest to the public health, epidemiology, and disease ecology communities that careful biodiversity documentation is critical to full description of disease research. As a routine part of the research process, voucher specimens should be deposited in an appropriate scientific collection, and catalog numbers reported in publications,

Disease studies that lack careful biodiversity documentation are numerous, even in the recent literature. In the August 2009 issue of this journal, I found at least 4 articles that report sampling of hosts or vectors, yet make no mention of vouchers (*4*–*7*). Quite simply, and with few counterexamples (*8*), host and vector information is ignored, as if all identifications are perfect and complete and as if nothing remains to be learned from further study of the samples.

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The reality, however, is quite different. First, technologies for diagnosis and testing have evolved considerably and will continue to evolve, with each iteration providing more complete information and insight into the pathogens present. The failure to preserve voucher specimens, however, makes such retesting and improved learning impossible. For example, in early studies of filoviruses, thousands of specimens were tested serologically for evidence of infection (*9*), with no positive results (*10*). However, new techniques would likely recover viral genetic material from those same samples (*11*), which could save time and expense invested in de novo sampling. Second, much remains to be learned from relationships between host population genetic structure and pathogen distributions. For example, some of the complexity of the distribution of Lassa fever depends on the particular lineage of *Mastomys* rodents present (*12*). Many host and vector groups currently considered single species are, in reality, complexes of species with potential (and possibly variable) epidemiologic importance. Such complexities can be explored only with detailed documentary information regarding which hosts did and did not harbor the pathogen.

Finally, and perhaps most urgent, treating biodiversity samples as disposable ignores opportunities to assemble archives of diagnostic samples for future studies. Host samples accumulated for 1 purpose could be recycled to form a strong basis for future studies of pathogens not yet known. Consider, for example, that those same samples of mammals from Africa from the early filovirus studies could have enabled quick and detailed study of *Henipavirus* distributions, in contrast to the time and effort it took to assemble other samples (*13*). Similarly, mammal samples assembled for early virus studies in West Africa (*14*) could have made possible rapid testing and evaluation of hosts for subsequent virus emergences in the region. In this sense, every biodiverse element collected as part of disease studies should be considered as potential key in-

frastructure for future studies, if properly documented and preserved (*15*).

Of course, biologic material that is potentially infected with dangerous pathogens carries with it some degree of responsibility, to ensure that unfortunate accidents do not occur. Two general paths are possible: 1) treatment of voucher specimen material to inactivate pathogens, such as preservation in formalin; or 2) notification by disease specialists to biodiversity specialists of any detections of pathogen-positive samples, such as samples that are inactivated or isolated. These steps are crucial, but the first option offers a way to avoid problems immediately with little extra effort.

My suggestion is not an empty dream but rather an open door. The biodiversity science community is fully prepared and willing to partner with the disease community in this effort. On the most proximate level, biodiversity specialists are eager to build scientific reference collections and are willing to curate and catalog voucher specimens. Vouchers provide permanent specimen identifiers that can be reported in publications and used to reference the voucher in genomic data bases. Furthermore, biodiversity specialists are interested in many of the same geographic regions as disease specialists and would welcome opportunities to obtain new specimen material from these regions. Finally, the role of pathogens in constraining host evolution, distribution, and ecology is of increasing interest in the biodiversity community (*16*,*17*). Many biodiversity researchers are extremely eager to explore new knowledge realms with disease specialists.

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Perinatal Pandemic (H1N1) 2009 Infection, Thailand

To the Editor: Infection with influenza A pandemic (H1N1) 2009 has been reported worldwide following initial identification of the virus in April 2009 (*1*). The groups at highest risk for infection or influenza-related complications include pregnant women and children (*2*). We report a case of pandemic (H1N1) 2009 infection in a newborn whose mother became ill with pandemic (H1N1) 2009 during the perinatal period.

A newborn girl showed signs of respiratory distress. The relevant perinatal history was maternal illness with pandemic (H1N1) 2009 7 days before delivery. The infant, who had a birth weight of 1,560 grams, was delivered by emergency cesarean section after the mother experienced cardiopulmonary failure at the gestational age of 31 weeks. Apgar scores were 9 and 9 at 1 and 5 minutes, respectively. Physical examination at birth showed a premature infant girl with mild subcostal retraction. Oxygen saturation at room air was 91%–99%. Other results of the physical examination were unremarkable.

Initial management included routine care for premature infants. On the basis of the perinatal history, a throat swab specimen was collected for pandemic (H1N1) 2009 testing by PCR and oseltamivir, 6 mg, was administered every 12 hours (4 mg/kg/day). The specimen obtained from the throat swab was positive for pandemic (H1N1) 2009 by real-time PCR. The infant required oxygen supplementation. At day 2 of life, acute renal failure with an elevated plasma creatinine level of 1.1 mg/dL developed in the infant. Chest radiograph showed minimal pulmonary infiltrations. She was started on cefotaxime for suspected sepsis. Oseltamivir dosage was adjusted based on the glomerular filtration rate estimated by the formula of Schwartz et al. (*3*) of 10.5 mL/min/1.73m^2 to 3 mg every 12 hours to complete 10 doses (2 mg/kg/day).

Infection of the patient was confirmed by real-time reverse transcription–PCR of the throat swab specimen and by a 4× increase in antibodies against the virus by hemagglutination inhibition test (HI). Antibody titers against pandemic influenza (H1N1) 2009 by HI with turkey erythrocytes (*4*) on days 10, 24, and 42 of life were 10, 160, and 320, respectively (Figure). At day 4 of life, repeated PCR performed on a throat swab specimen was negative for pandemic (H1N1) 2009. Oxygen supplementation was gradually decreased and finally discontinued. Her room air oxygen saturation was 95%–98%. Her clinical symptoms gradually improved. Hemoculture was negative after 72 hours. The antimicrobial drugs were given over an 8-day course. Plasma creatinine decreased to 0.9 mg/dL and 0.6 mg/dL at days 6 and 7 of life, respectively. Her average urine output was 2–3 mL/kg/h. She was discharged at the age of 28 days with a body weight of 2,070 grams.

Pregnant women are one of the highest risk groups for influenza A infection and influenza-associated complications, including increased maternal and perinatal illness and death rates (*5*). Thus, pregnant women are given first priority to receive influenza vaccination. When influenza develops in mothers during the perinatal period, newborns can be infected transplacentally during maternal viremia or by respiratory droplets after birth. Transplacental infection of influenza A is rare (*5*), however, and there have been only a few case reports (*6*,*7*). Viremia is more frequent and more extensive in pregnant women due to depressed cell-mediated immune response during the pregnancy (*8*). Our patient was likely infected in utero because she was delivered by cesarean section and was never exposed to her mother, who required intensive cardiopulmonary support at the time of delivery. (The mother died from respiratory failure 7 days after the cesarean section.)

Clinical manifestations in this patient, including respiratory distress and acute renal failure, were nonspe-

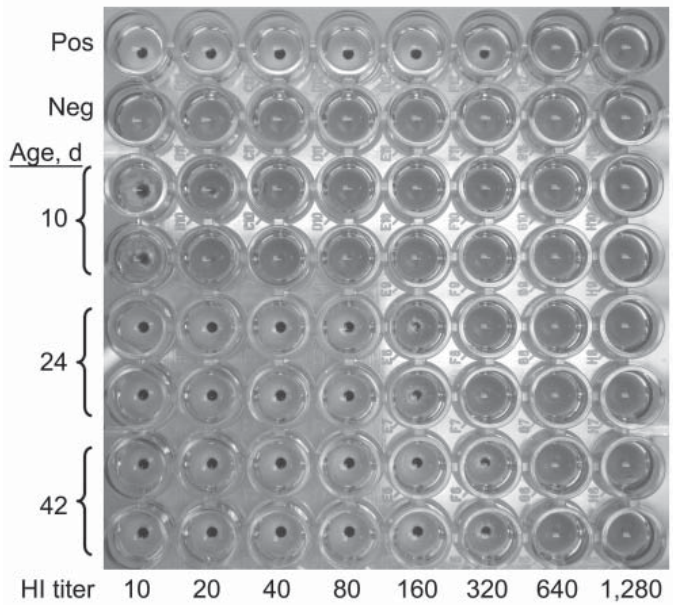

Figure. Antibody titer against influenza A pandemic (H1N1) 2009 by hemagglutination inhibition (HI) test on days 10, 24, and 42 of life of the patient. A color version of this figure is available online (www.cdc.gov/EID/content/16/2/343-F.htm).

cific. The high plasma creatinine level in the newborn sometimes reflects the mother's plasma creatinine level (*9*). However, kidney function of the mother of the newborn was within normal limits at the time of Caesarean section; plasma creatinine level of 0.7 mg/dL. An elevated plasma creatinine level is observed frequently in premature infants due to immaturity of the kidney tissue and will usually decrease within a few weeks. Oseltamivir was administered with dose adjustment based on the infant's estimated glomerular filtration rate**.** The recommended dose of oseltamivir for glomerular filtration rate <30 mL/min/1.73 m^2 is 2–3 mg/kg/day, based on preliminary data obtained by a National Institutes of Health–funded Collaborative Antiviral Study Group (*10*). The success of our management strategy for this case suggests early treatment with oseltamivir can prevent severe illness in newborns with perinatal influenza A pandemic (H1N1) 2009 infection.

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Bronchial Casts and Pandemic (H1N1) 2009 Virus Infection

To the Editor: In the late 1990s, triple-reassortant influenza A viruses containing genes from avian, human, and swine influenza viruses emerged and became enzootic in swine herds in North America (*1*). The first 11 human cases of novel influenza A virus infection were reported to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) from December 2005 through February 2009 (*1*). In response to those reports, surveillance for human infection with nonsubtypeable influenza A viruses was implemented.

In the spring of 2009, outbreaks of febrile respiratory infections caused by a novel influenza A virus (H1N1) were reported among persons in Mexico, the United States, and Canada (*2*). Patient specimens were sent to CDC for real-time reverse transcription–PCR (RT-PCR) testing, and from April 15 through May 5, 2009, a total of 642 infections with the virus, now called pandemic (H1N1) 2009 virus, were confirmed. Of those 642 patients, 60% were ≤18 years of age, indicating that

children may be particularly susceptible to pandemic (H1N1) 2009 (*2*).

Children and adults with preexisting underlying respiratory conditions, such as asthma, are at increased risk for complications from infection with pandemic (H1N1) 2009 virus. One possible complication is plastic bronchitis, a rare respiratory illness characterized by formation of large gelatinous or rigid branching airway casts (*3*). Plastic bronchitis is a potentially fatal condition induced by bronchial obstruction from mucus accumulation resulting from infection, inflammation, or vascular stasis (*4*). We report a case of bronchial casts that caused atelectasis of the right lung of a child infected with influenza A pandemic (H1N1) 2009 virus.

A 6-year-old boy with asthma and a 1-day history of fever and cough was referred to a hospital pediatrics department because of dyspnea. Clinical examination at hospital admission found respiratory distress, as shown by tachypnea (respiratory rate 66 breaths/min) and inspiratory retraction, deficient vesicular sounds over the right lung field, elevated blood levels of immunoglobulin E (1,770 IU/mL) and a reduced number of lymphocytes (483 cells/μL), and radiographic evidence of atelectasis of the right lung and hyperinflation of the left lung without air leakage (Figure, panel A). Pandemic (H1N1) 2009 virus infection was confirmed by real-time RT-PCR, as described (*5*), of an endotracheal aspirate. Real-time PCR ruled out *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Legionella pneumophila, Chlamydophila pneumoniae, S. pyogenes*, respiratory syncytial viruses A and B, seasonal influenza viruses A and B, parainfluenza viruses 1–3, rhinovirus, enterovirus, human metapneumovirus, human bocavirus, and adenovirus (*6*). While the patient was breathing room air, his percutaneously monitored oxygen saturation was 86%; respiratory support by mechanical ventilation was then initiated. Mucus casts were extracted by intratracheal suction (Figure, panel B). The patient was treated with an inhaled bronchodilator, intravenous methylprednisolone (20–60 mg/day for 7 days), and antiviral (oseltamivir) and antimicrobial (ampicillin/sulbactam) drugs.

On hospital day 2, chest radiographs showed that atelectasis of the right lower lobe had partially resolved (Figure, panel C). A histologic examination of casts (May-Giemsa stain; Figure, panel D) indicated a mucoid substance containing a predominantly eosinophilic infiltrate (>90% of cells). The patient's respiratory condition during 11 days of oxygen supplementation gradually improved, and he was discharged on hospital day 18.

Plastic bronchitis is related mainly to respiratory, cyanotic cardiac (post-Fontan), and hematologic (sickle cell anemia) diseases. A diagnosis of plastic bronchitis is determined on the basis of clinical findings (pointing to allergic and asthmatic, cardiac, or idiopathic etiologies) and pathologic findings (inflammatory vs. noninflammatory) on examination of casts (*3*). Inflammatory casts contain fibrin, eosinophils, and Charcot-Leyden crystals; noninflammatory casts contain mucin and exhibit vascular hydrostatic changes. The case presented here was the allergic-inflammatory type of plastic bronchitis.

Various treatments for plastic bronchitis have been described and vary from cast removal by expectoration or by bronchoscopy (*7*,*8*). Other interventions involve cast disruption by tissue plasminogen activator or urokinase and prevention of cast formation by use of mucolytic agents, steroids, or anticoagulants. However, evidence remains anecdotal because too few plastic bronchitis patients are available for clinical trials. Details of steroid dosage will need to be clarified for pandemic (H1N1) 2009 virus–infected children with respiratory distress from bronchitis and pneumonia.

In Iran during 1998–2001, avian influenza (H9N2) infection among broiler chickens resulted in 20%–60% mortality rates on affected farms (*9*). Macroscopic examination of specimens from infected chickens showed extensive hyperemia of the respiratory tract, followed by exudate and casts extending from the tracheal bifurcation to the secondary bronchi. Light microscopy indicated severe necrotizing tracheitis. Pandemic (H1N1) 2009

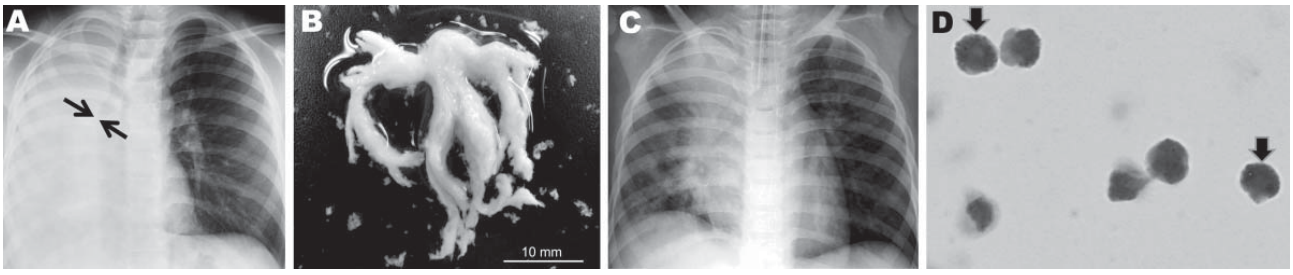

Figure. A) Chest radiograph obtained at hospital admission from a child infected with influenza subtype H1N1 virus. The image shows atelectasis of the right lung and hyperinflation of the left lung; arrows indicate obstruction of the right main bronchus. B) Macroscopic bronchial casts extracted by intratracheal suction. C) Chest radiograph obtained on hospital day 2, indicating partial resolution of atelectasis of the right lower lobe. D) Light micrograph of casts, characterized by predominant eosinophil infiltration (>90% of cells) (May-Giemsa stain, original magnification ×1,000). Arrows indicate typical eosinophil granules. A color version of this figure is available online (www.cdc.gov/EID/content/16/2/344-F.htm).

can produce similar airway cast formation in humans; severe respiratory distress reflects extensive obstruction of the respiratory system.

Healthcare providers should be aware of the possibility of bronchial casts when examining children with influenza (H1N1) infection accompanied by atelectasis. Steroids can be administered early in infection to avoid cast formation, and antiviral drug therapy and respiratory support can be used for influenza (H1N1)–infected children in whom airway casts have developed.

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Methicillin-Resistant *Staphylococcus aureus* ST398, Italy

To the Editor: It has recently become apparent that livestock can constitute a new methicillin-resistant *Staphylococcus aureus* (MRSA) reservoir and be a source of a novel and rapidly emerging type of MRSA. These livestock-associated MRSA clones are nontypeable by use of pulsed-field gel electrophoresis with *Sma*I and belong to sequence type (ST) 398 (*1*). MRSA ST398 clones account for 20% of all MRSA in the Netherlands (*2*), but the emergence of such clones has been described worldwide (*3*). Although ST398 transmission has been reported primarily between animals, persons with occupational exposure to livestock are at higher risk for MRSA carriage than the general population. Even though MRSA ST398 usually causes colonization, several cases of infections of variable clinical relevance, varying from skin and soft tissue infections (*4*) to endocarditis (*5*) and pneumonia (*6*), have been described over the past few years. Most instances of ST398 human carriers have been identified among persons who work at pig farms (*7*). Data regarding MRSA colonization of dairy farmers are less exhaustive and, to our knowledge, only 1 instance of direct transmission between cattle and humans has been proven. MRSA isolates from cows with subclinical mastitis in 2007 in Hungary were indistinguishable from MRSA isolates from the tonsil swab of a farmer who worked with these animals (*8*). We report a case of MRSA ST398 invasive disease in a cattle farmer, as well as a case of MRSA ST398 necrotizing fasciitis.

In early April 2008, a 52-year-old man was admitted to an intensive care unit in Manerbio, Italy, because of severe sepsis and a large ulcerative and

suppurative lesion on the right side of his neck. His medical history was unremarkable. He was a worker at a dairy farm, was obese, and did not report any previous contact with the healthcare system. At the time of hospital admission, he was oriented and cooperative. His temperature was 38.4°C, heart rate was 125 beats per minute, and blood pressure was 165/75 mm Hg. Arterial blood gas analysis showed hypoxemia and mild hypocapnia (PaO_2 53 mm Hg and $PaCO_2$ 33.8 mm Hg on room air). Leukocyte count was 21,280 cells/μL (81.9% polymorphonuclear cells), and platelet count was 310,000 cells/μL. After blood samples were collected and aggressive surgical debridement of affected tissue was performed, empirical treatment with intravenous teicoplanin and imipenem was started. On the basis of histologic appearance of the intraoperative material and computed tomography scan images, necrotizing fasciitis was diagnosed. Culture of blood and necrotic tissue yielded MRSA. On day 3 after admission, antimicrobial drug therapy was changed to teicoplanin and clindamycin and, on day 7, to linezolid. Fever resolved in 3 days and the patient's condition progressively improved. The patient was discharged after 31 days of antimicrobial drug therapy. The MRSA isolate was susceptible to all the non–β-lactam antimicrobial drugs tested (excluding tetracycline), carried the staphylococcal cassette chromosome *mec* type V, and was negative for Panton-Valentine leukocidin (PVL) genes. Multilocus sequence typing and sequence typing of the tandem repeat region of protein A gene (*spa* typing) showed that the isolate belonged to ST398 and *spa* type 899, respectively.

Some issues are of concern. Although the MRSA isolate was PVL negative, its virulence resembled that of PVL-positive strains. Furthermore, it was resistant to tetracycline, as we expected because oxytetracyclines are the antimicrobial drugs most frequently used in pig and cattle farming (*3*). The major limitation of our study was that data regarding MRSA colonization of the farm are missing, so cattle-to-human transmission cannot be proven. However, because our patient did not have any other potential risk factor, dairy cows were probably the source of the human infection. In countries where community-acquired MRSA is common, all patients with serious *S. aureus* infections should be treated for MRSA until antimicrobial susceptibilities are known. Our report suggests that even in countries where community-acquired MRSA is still rare, being a cattle farmer may be considered an indication for early treatment against MRSA.

The expanding knowledge of this zoonotic potential may undermine existing nosocomial MRSA control programs. In countries where a search and destroy policy (*9*) is adopted, such as the Netherlands, pig and cattle farmers may warrant screening and isolation at the time of hospital admission. Nevertheless, the first MRSA ST398 nosocomial outbreak has already been described (*10*).

It is difficult to prevent persons with constant exposure to MRSA in their work or home setting from becoming MRSA carriers. Revisiting policies for the use of antimicrobial drugs on livestock farms, as well as improving hygiene measures, may therefore be necessary in infection control programs. However, before final recommendations can be made, further investigation is needed to determine the prevalence of MRSA among livestock and their handlers.

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Neisseria meningitidis Serogroup W135, China

To the Editor: *Neisseria meningitidis* is a gram-negative bacterium found only in humans and is a major cause of serious invasive diseases. Before 2006, in the People's Republic of China, all meningococcal diseases were caused by serogroups A, B, and C. However, there are ≥13 serogroups of this organism. Three cases of infection with *N. meningitidis* serogroup W135 were reported in China during 2006–2008. We describe these 3 meningitis patients and the *N. meningitidis* serogroup W135 strains isolated from these patients by genotyping methods.

Patient 1, a 36-year-old man, was seen at a local hospital in Fujian Province in January 2006. He became ill while on a business trip and was given a diagnosis by culture of an *N. meningitidis* infection. Patient 2, a 25-year-old man, was seen in Guangdong Province in May 2007. He had not traveled outside this area in the 10 days before becoming ill. Patient 3, a 14-year-old girl, was seen in Guangxi Province in February 2008. She was a middle school student and had toured the suburbs of this province with her classmates 2 days before becoming ill. Close contacts of all 3 patients were investigated; no additional *N. meningitidis* infections were detected. However, *N. meningitidis* was isolated from a throat swab specimen obtained from the younger cousin of patient 3.

N. meningitidis infection was confirmed for all 3 patients on the basis of clinical symptoms and laboratory results. All patients reported neck stiffness. Physical examinations showed Kernig signs, Brudzinski signs, and high temperatures (>38°C). Cerebrospinal fluid (CSF) samples were turbid with increased protein levels and pressure; leukocyte counts were increased (>5,000 cells/μL). CSF culture on chocolate agar grew *N. meningitidis* after 24 h. Isolates were identified as serogroup W135 by using specific antiserum (Remel, Lenexa, KS, USA) at provincial Centers for Disease Control and Prevention (CDC) in China and confirmed at the Chinese CDC.

Patients were treated with antimicrobial drugs and recovered fully. An isolate from the cousin of patient 3 was also identified as W135. Etest strips and broth microdilution were used for antimicrobial drug susceptibility testing for the 4 W135 isolates. All isolates were susceptible to 12 antimicrobial drugs tested, which included therapeutic and prophylaxis agents used frequently in China.

Pulsed-field gel electrophoresis (PFGE), multilocus sequence typing, and outer membrane protein (*por*A) gene variant region subtyping were used to characterize the 4 case-related W135 *N. meningitidis* isolates and other isolates from asymptomatic carriers. Strain R29057 (from France) was used as a reference strain. The 4 case-related isolates showed similar PFGE patterns. These patterns were distinct from those of other W135 isolates obtained from asymptomatic carriers. Three invasive disease isolates and 1 from the close contact of patient 3 had the same multilocus sequence type (ST) and PorA subtype; all were ST11: P1.5, 2. This subtype was not detected among other tested isolates of W135 obtained from asymptomatic carriers (online Technical Appendix, www.cdc.gov/EID/content/16/2/348-Techapp.pdf).

ST11: P1.5, 2 *N. meningitidis* serogroup W135 was responsible for the epidemic of W135 meningococcal disease in 2000, which was associated with the Hajj pilgrimage in Saudi Arabia (*1*,*2*). The strain related to the Hajj pilgrimage was derived from clonal expansion within the ST11 complex/ET-37 complex (*3*). However, no epidemiologic data showed that the 3 cases in our study were linked to the Hajj pilgrimage. Since 2000, invasive diseases caused by W135 meningococci of ST11 have been reported in Africa, Asia, and the Middle East (*4*). ST11 W135 infections have been reported to cause invasive disease in Taiwan during 1996–2002 and were apparently introduced into Taiwan before the Hajj pilgrimage–associated outbreak because they were genotypically distinct from the Hajj-related W135 clone (*5*,*6*).

The 3 cases we report were observed in southeastern China near Taiwan (online Technical Appendix), but no direct epidemiologic links are known. Because of the lack of W135 strains from Hajj pilgrimages and Taiwan in this study, we could not provide a detailed and integrated genotypic relationship between the strains in China and those of Hajj pilgrimages and Taiwan. However, we can confirm that these 3 cases were caused by strains from the same hypervirulent clone characterized as ST11: P1.5, 2.

W135 strains have been isolated after vaccination with a bivalent meningococcal vaccine in Cameroon (*7*). In China, the bivalent meningococcal vaccine has been successfully introduced into the national expanded immunization program in response to an outbreak of *N. meningitidis* serogroup C during 2003–2004 (*8*).

The 3 patients infected with W135 in our study did not receive bivalent meningococcal vaccines. W135 meningococcal disease appears to be an emerging problem that should be investigated epidemiologically. These patients highlight the need for further epidemiologic surveillance to monitor changes in the incidence of meningococcal disease caused by W135 and for improved public health disease control strategies in the future.

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Avian Influenza (H5N1) Outbreak among Wild Birds, Russia, 2009

To the Editor: Highly pathogenic avian influenza (HPAI) virus (H5N1) has been endemic in poultry in Southeast Asia since 2003 (*1*). In April 2005, an outbreak of influenza virus (H5N1) infection was detected in wild birds on Qinghai Lake in western China (*2*). Subsequently, the Qinghai-like (clade 2.2) HPAI virus (H5N1) lineage was detected in wild birds and poultry in many countries (*1*,*3*,*4*). The source of these introductions, although still debated, is likely through bird migration (*5*).

In June 2006, an influenza (H5N1) outbreak was detected in wild birds on Uvs-Nuur Lake in western Siberia, Russia. We showed that A/duck/Tuva/01/2006, isolated during the outbreak, was highly pathogenic for chickens and mice and belonged to the Qinghai-like group (2.2 clade) (*6*).

The first case of Fujian subclade 2.3.2 influenza virus (H5N1) lineage in the Russian Far East was recorded in April 2008 (*7*). Before this case, no HPAI (H5N1) outbreaks of the Fujian lineage had been reported in Russia.

In June 2009, an outbreak of HPAI in wild birds was recorded in Mongolia (*4*) and on Uvs-Nuur Lake in Russia. RNA extracted from organs (liver, spleen, intestine) of 10 dead birds belonging to 4 species (great crested grebe [*Podiceps cristatus*], little grebe [*Tachybaptus ruficollis*], black-headed gull [*Larus ridibundus*], and spoonbill [*Platalea leucorodia*]) was positive for type A influenza RNA and for the H5 subtype by real-time reverse transcription–PCR (*8*). We isolated 2 viruses from embryonated specific antibody–negative fowl eggs. Hemagglutination (HA) and neuraminidase (NA) inhibition assays with monospecific antiserum confirmed the H5N1 subtype. Viruses were designated as A/black-headed gull/Tyva/115/2009 and A/great crested grebe/Tyva/120/2009, and sequences of their HA and NA segments were defined. No HPAI virus (H5N1) was found in cloacal swabs obtained from 36 live birds (of the 4 species listed above) from Uvs-Nuur Lake.

Phylogenetic analysis (*9*) of the HA gene (Figure) showed that viruses belong to clade 2.3.2. These viruses are clearly distinguishable from the HPAI viruses previously isolated in this Russian region in 2006, A/duck/Tuva/01/2006 (clade 2.2) but are more related to A/whooper swan/Mongolia/8/2009 and A/whooper swan/Mongolia/2/2009. For the NA

gene, isolated viruses were most closely related to viruses found in Mongolia. Analysis of NA protein determined that the viruses found are sensitive to NA inhibitors.

Both viruses were shown to be highly pathogenic for chickens (intravenous pathogenicity index 3). This finding is consistent with the results of the sequence analysis of the HA gene. The HA protein possesses a series of basic amino acids (PQRERRRKR) at the cleavage site. Several amino acid changes were found between HA of investigated viruses and viruses from clade 2.3.2 that were isolated in Russia in 2008. However, the receptor-binding site of HA (positions 222–224) was not changed.

The spread of HPAI (H5N1) west across the globe has caused serious debates on the roles of migratory birds in virus circulation (*2*,*5*,*7*). In the 2009 outbreak we describe, we doubt that wild birds were infected from local poultry because domestic poultry are not present in the Uvs-Nuur Lake region and there have been no reports of HPAI among poultry in Russia since early 2008. We suggest that wild birds brought the virus to Uvs-Nuur Lake from outside the country. Because prior to June 2009 the only case of new Fujian sub-clade 2.3.2 influenza virus (H5N1) lineage was in the Russian Far East, we believe that the virus isolated in 2009 from Uvs-Nuur Lake was probably introduced by wild birds that wintered in Southeast Asia.

Many different bird species stop at Uvs-Nuur Lake during the spring and fall migrations. Qinghai-like viruses were introduced to the region from central China by wild birds in 2006 (*6*). The introduction of the H5N1 Fujian-lineage to the lake 3 years later shows further evidence that Uvs-Nuur Lake is an major area for wild bird migration and breeding and hence an environment that could potentially support the introduction of influenza virus variants from migrating wild birds. Bodies of water such as Qinghai Lake and Uvs-Nuur Lake may play a major role in the circulation of avian influenza. Therefore, we continue to study new outbreaks thoroughly and take into account the ecology and pathobiology of the species involved. Areas where large numbers of birds congregate should be closely monitored because these areas could serve as the breeding ground for avian influenza virus variants that might spread globally. Additionally, we must keep in mind that wild bird species can vary greatly in their response to HPAI and that naturally resistant waterfowl could serve as vectors for the introduction of HPAI into new locations (*1*,*2*,*5*,*7*).

Because wild birds can be involved in virus introduction, continuing surveillance is warranted. Detection of any influenza A (H5N1) virus in wild birds in a new region should be immediately followed up with efforts to characterize the virus and to control the spread of new HPAI viruses.

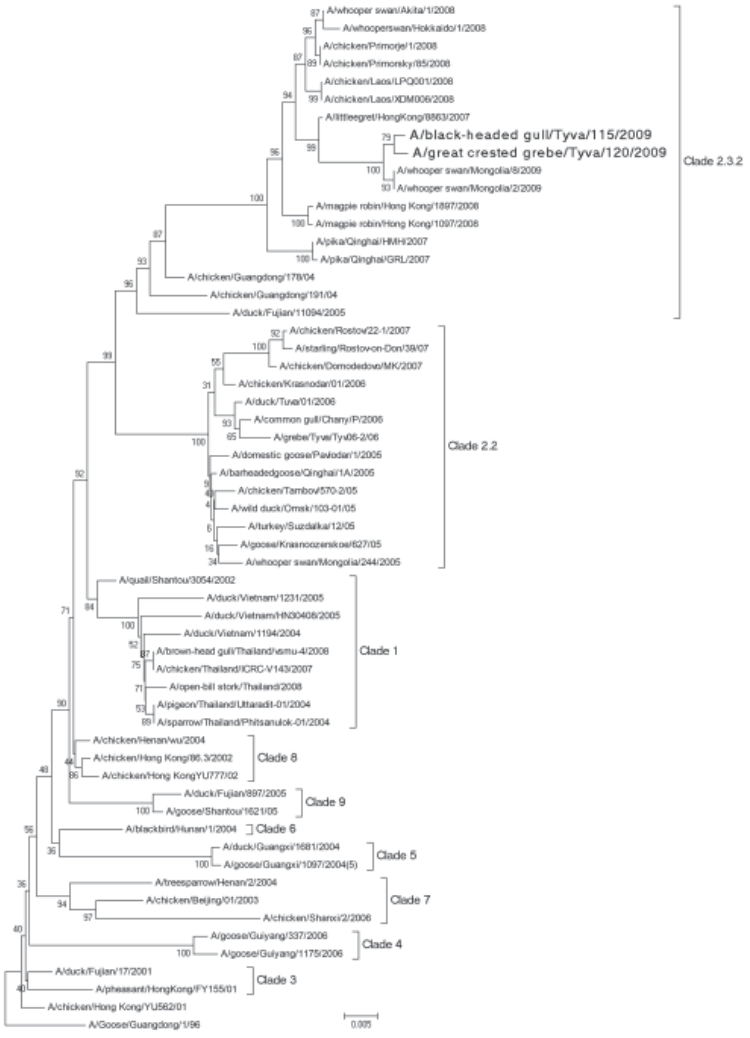

Figure. Phylogenetic tree constructed by neighbor-joining analysis (no. replications ×600) of the hemagglutinin gene segment of representative influenza virus (H5N1) isolates. Taxon names of the viruses isolated in Russia in 2006 and 2009 are in **boldface**. Scale bar indicates genetic distance.

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Detection of Pandemic (H1N1) 2009 Virus in Patients Treated with Oseltamivir

To the Editor: In April 2009, an influenza outbreak caused by a novel strain of influenza virus A (H1N1) was identified in Mexico. The rapid spread of this new virus among humans led the World Health Organization to raise the phase of pandemic alert to 6. We report results from the 2 virology laboratories from university hospitals that were involved in the surveillance network of pandemic (H1N1) 2009 in Paris at the beginning of the outbreak in France.

Patients exhibiting influenza-like illness (i.e., fever, sore throat, cough, asthenia, headache, myalgia) and who recently had traveled to countries where the pandemic (H1N1) 2009 outbreak had started (i.e., Mexico, United States, Canada, Japan) were hospitalized. Symptoms began either the day before or the day of hospitalization. Nasal-swab specimens were collected at admission by using the Virocult system (ELITech; Salon-de-Provence, France), and treatment with oseltamivir was started (75 mg, 2×/day). Pandemic (H1N1) 2009 infection was diagnosed by using rapid test QuickVue Influenza A+B (Quidel, San Diego, CA, USA) and real-time reverse transcription–PCR (RT-PCR) assays from the French National Influenza Centers or the US Centers for Disease Control and Prevention (*1*). In the case of a positive result, influenza virus in nasal secretions from patients was monitored daily by RT-PCR until viral genomes became undetectable.

From April 24 through June 7, 2009, nasal swab specimens from 234 persons (132 men; median age of all patients 33 years) were processed; pandemic (H1N1) 2009 infection was confirmed for 17 men and 15 women (median age 33 years) by RT-PCR. Results of the Quidel rapid tests were available for 27 specimens, with positive results for 9 (33% sensitivity). However, no positive result was observed with the Quidel rapid tests among the nasal swab specimens with negative RT-PCR results (100% specificity). Influenza virus detection in nasal secretions was monitored for 16 patients who had laboratory-confirmed pandemic (H1N1) 2009 infection and were treated with oseltamivir. Viral detection by RT-PCR was absent 2 to >5 days after antiviral treatment began (Figure). Significant differences were not found in sex and age of the patients (data not shown).

These preliminary virologic data obtained during the first 6 weeks of pandemic (H1N1) 2009 in France confirm the poor sensitivity of the Quidel test toward this new virus, as recently reported (*2*). Further studies are needed to evaluate the performances of other rapid tests. Hayden et al. (*3*) demonstrated that treatment with oseltamivir significantly reduced duration of viral shedding among patients infected with seasonal influenza virus A (H1N1), in comparison with a placebo group: 1.5–2.5 days vs. 3.5–5.5 days ($p = 0.003$). In our study, surprisingly, PCR results for sequential nasal swab specimens from 16 patients infected by pandemic (H1N1) 2009 and treated with oseltamivir were negative within 3 days after therapy for only 9 (56%); indeed, for 3 (19%) patients, viral genome could be detected >5 days after antiviral treatment began. These data raise questions about potential virus transmission during antiviral treatment and the possible resistance of

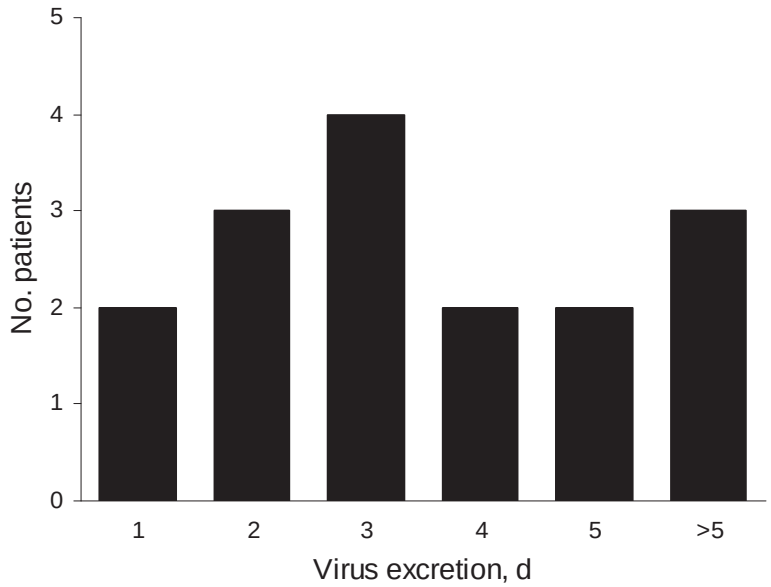

Figure. Duration of pandemic (H1N1) 2009 excretion in nasal swabs from patients treated with oseltamivir. The number of days from start of oseltamivir treatment to achievement of negative results of reverse transcription–PCR (RT-PCR) is indicated for 16 patients. The 3 patients classified in the last group (>5 days) are 1 patient with a negative RT-PCR result on day 7 posttreatment and 2 patients who still had positive results on day 5 posttreatment but provided no additional sample for testing.

pandemic (H1N1) 2009 to oseltamivir. This latter point is now under study.

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Marburg Virus in Fruit Bat, Kenya

To the Editor: Lake Victoria Marburgvirus (MARV) causes severe hemorrhagic fever with a high case-fatality rate in humans. Index cases occurred in Europe during 1967 among laboratory workers who handled tissues and blood samples of nonhuman primates from Africa (*1*). Thereafter, MARV was reported throughout sub-Saharan Africa. Most outbreaks in humans were associated with visits to caves and mines (*2*–*6*). In Kenya, human cases of MARV infection were reported in 1980 and 1987; these occurred after visits to the Kitum Cave at Mount Elgon (*7*,*8*). MARV was detected in tissues of Egyptian fruit bats (*Rousettus aegyptiacus*) and other bat species from the Democratic Republic of Congo (DRC), Gabon, and Uganda (*3*–*6*).

We collected bats from across Kenya during June–July 2007 within the framework of the Global Disease Detection Program, which is dedicated to investigation of emerging pathogens. Collection protocols were approved by the National Museums of Kenya and by the Centers for Disease Control and Prevention (Atlanta, GA, USA). Blood, fecal and oral swab specimens, and selected tissue samples were collected from bats and stored on dry ice.

For MARV detection, total RNA was extracted from pooled or individual liver, spleen, and lung samples from 272 bats. Nested reverse transcription–PCR (RT-PCR) with primers specific for MARV nucleoprotein gene was performed as described (*5*). When a band of the expected size was detected after electrophoresis on an agarose gel, the RT-PCR product was sequenced. Laboratory cross-contamination was not a concern because no work with MARV had been conducted in the facility where the examination was performed.

MARV RNA was detected in pooled liver, spleen, and lung tissue of an apparently healthy, pregnant,

female *R. aegyptiacus* bat obtained at Kitum Cave in July 2007 (Figure). A faint band was obtained only after nested RT-PCR, which suggests that the RNA load was limited. Attempts at virus isolation were not performed. Phylogenetic comparisons demonstrated that the virus (KE261, GenBank accession no. GQ499199) was relatively distant from previous isolates from Kenya (Musoke and Ravn). It was similar to viruses isolated from index cases in Europe in 1967 (Popp and Ci67). This lineage also contained virus 02DRC99, which was isolated from a human in the DRC in 1999 (online Appendix Figure, www.cdc.gov/EID/content/16/2/352-appF.htm). MARV isolates obtained from bats and humans in Uganda in 2007 belong to distinct lineages (*6*) (online Appendix Figure). Tissues of other bats, including 75 *R. aegyptiacus* (29 pregnant females) from Kitum Cave and neighboring Makingeni Cave, were negative for MARV RNA.

Histopathologic examination of liver of the infected bat showed no lesions that could be ascribed to MARV infection, and no MARV antigens were detected by immunohistochemical analysis. Other tissues were not examined.

Our results are similar to those reported from Gabon and the DRC, where MARV RNA was detected in tissues of 1.4% and 3.1% of *R. aegyptiacus* bats, respectively, with negative isolation attempts (*3*,*5*). A higher prevalence (5.1%) was detected in *R. aegyptiacus* bats from Uganda in 2007, where several MARV isolates were obtained from bats with high virus loads (*6*). In the DRC, MARV RNA was also detected in insectivorous bats, including 3.0% of *Miniopterus inflatus* and 3.6% of *Rhinolophus eloocuens* (*3*,*5*). However, in Uganda, MARV RNA was detected in only 1 (0.2%) of 609 insectivorous bats (*Hipposideros* spp.) (*6*).

To date, bats are the only wild mammals, besides nonhuman primates, in which filoviruses have been detected. Whether bats serve as principal reservoir hosts for filoviruses is unclear. The pathogenesis and clinic manifestation of filovirus infection in bats are unknown. Colonies of *R. aegyptiacus* bats in caves often consist of thousands of bats. The opportunity for conspecific exposure rates in such colonies is high. Therefore, bat populations should have a high seroprevalence rate for these viruses. For example, seroprevalence to lyssaviruses in some bat species that live in colonies was reported as high as 60%–70% (*9*). In contrast, seroprevalence of MARV-neutralizing antibodies in colonies of *R. aegyptiacus* bats in which PCR-positive bats were collected was only 12% (*5*) or as low as 2.4% (*6*). This low seroprevalence may be interpreted as a result of a limited spillover of MARV into bats from another source.

The association of human cases of MARV with visiting caves often inhabited by *R. aegyptiacus* and other bat species is obvious (*3*,*5*,*6*). This association was reinforced by MARV infection in tourists who visited caves in Uganda (*4*,*10*). For Kenya, our finding is consistent with reported human cases tentatively associated with visiting of Kitum Cave (*7*,*8*). We do not know if MARV has persisted in this area continuously or has reemerged sporadically. Kitum Cave and other similar caves are easily accessible and frequently visited by tourists and local persons. The likelihood of MARV spillover into humans is presently limited. However, because transmission mechanisms and sources of spillover infections are unknown, public awareness must be increased and health authorities informed about the presence of MARV.

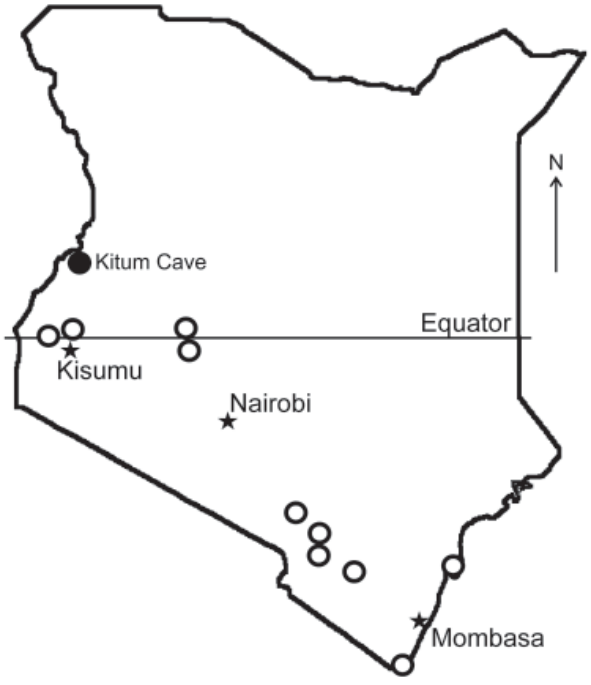

Figure. Bat collection sites (open circles) and location of Kitum Cave, Kenya, where Lake Victoria Marburgvirus was detected (solid circle).

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Human African Trypanosomiasis in Areas without Surveillance

To the Editor: Human African trypanosomiasis (HAT), sleeping sickness, is a systemic protozoan disease transmitted by the bite of a tsetse fly; untreated infection is fatal (*1*). Control of HAT caused by *Trypanosoma brucei gambiense*, which caused 97% of all cases reported from 1997 through 2006 (*2*), is based on active screening of the population at risk by mobile teams and treatment of all infected persons, with or without vector control.

The epidemiologic curve of reported new cases varies considerably; incidence peaks were high in the 1920s and 1990s but low in the 1960s and in the past decade (2000–2009) (*2–4*). The recent reduction of reported cases (69% decrease from 1997 through 2006) was made possible by 1) cessation of large-scale civil wars (e.g., in Angola); 2) increased commitment of donors, national control programs, the World Health Organization (WHO), and nongovernment organizations; and 3) free production and supply of antitrypanosomal drugs. In May 2007, after a WHO informal consultation on sustainable sleeping sickness control, representatives from countries to which HAT is endemic concluded that HAT elimination is possible (*5*). Médecins sans Frontières (MSF), an international nongovernment organization, wishes to challenge this conclusion.

Because of insufficient coverage by surveillance systems, only a fraction of HAT cases are reported. In 2004, for example, although WHO received reports of only 17,500 new cases, they estimated that the actual incidence was 50,000–70,000 cases (*6*). Recent MSF HAT projects in remote and politically unstable areas of the Central African Republic and the Democratic Republic of Congo are finding new information about the location and nature of some of these blind spots (areas without surveillance) (Table).

In the zones de santé (administrative districts) of Doruma, Ango, and Bili, in northeastern Democratic Republic of Congo, no HAT control activities have taken place over the past 3 decades, mainly because of extreme remoteness of these areas. In July 2007, MSF launched a HAT control program and found high (3.4%) disease prevalence and a large proportion of patients in the first stage of the disease (60%), indicating intense transmission. In March 2009, the MSF team was attacked by rebels from the Lord's Resistance Army, leading to total suspension of the project for an indefinite period. The lack of trained staff in existing health structures and the complexity of HAT management prevented emergency handover of the project to local partners.

Table. Human African trypanosomiasis control programs run by Médecins sans Frontières in central Africa, January 2007–May 2009*

Location (program dates)	No. persons screened			No. (%) new diagnoses			Prevalence, %†
	Total	Passive	Active	Total	First stage	Second stage	
Zones de santé of Doruma, Ango and Bili, DRC (2007 Jul–2009 Mar)	46,601	18,559	28,042	1,570	947 (60)	623 (40)	3.4
Maitikoulou and neighboring villages, CAR and Chad (2009 Jan–2009 May)	4,633	286	4,347	665	450 (68)	215 (32)	14.4

*DRC, Democratic Republic of Congo; CAR, Central African Republic.
†Total no. patients with parasitologically confirmed human African trypanosomiasis (total no. new diagnoses) divided by total no. persons screened during the period of first-round active screening (total no. persons screened).

Batangafo and Maitikoulou, in northwestern Central African Republic, are also historical foci of HAT that have been neglected over the past years, mainly because of political insecurity and logistic constraints. From January 2007 through April 2009, HAT was diagnosed for 1,074 patients in Batangafo (prevalence 3.1%). From January through May 2009, MSF screening of 4,633 persons from 23 villages around Maitikoulou in Central African Republic and Chad found high disease prevalence and a high proportion of first-stage illness.

Because the case-finding activities in the Central African Republic and Democratic Republic of Congo were restricted for security reasons, the MSF programs may only be seeing a small part of the problem. The above-listed examples illustrate that many HAT patients are still found in historical foci that have been devoid of active surveillance for years or decades because of their remoteness, insecurity, neglect, or a combination of these factors. Thus, many more patients probably continue to have no access to care and therefore remain invisible.

We emphasize the crucial need to continue research and development efforts toward simpler diagnostic methods and treatment. The effect of inadequate tools is particularly obvious in remote or unstable areas of high disease prevalence, where health facilities are often poorly functioning and severely understaffed. The recent completion of a study showing excellent safety and efficacy of a simpler treatment for second-stage patients is an encouraging first step (*7*).

The remoteness of many HAT-endemic areas, persistence of forgotten conflicts, and insufficient resources continue to restrict the possibility of eliminating HAT, except in countries or regions where the disease is already well controlled or where control programs cover all disease-endemic foci. As long as most HAT patients continue to have no access to care and are therefore not reported, HAT cannot be eliminated. Moreover, countrywide statistics should be interpreted with caution. The decline of new cases observed in the Democratic Republic of Congo from 1998 through 2003 is largely the result of efforts of 1 nongovernment organization in 1 province (Equateur-Nord), while the incidence trends remained stable in other provinces (*8*).

Donors must be aware that HAT epidemiology is heterogeneous. The allocation of funds should not be restricted to maintaining surveillance and control efforts in areas of low disease prevalence (to prevent future flare-ups). Adequate funding must also be provided to allow control programs to reach remote disease-endemic foci that have been left without active surveillance for years.

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Using Museum Collections to Detect Pathogens

To the Editor: Natural history museum collections have evolved in recent years to meet the challenges of current and future interdisciplinary scientific studies. Many natural history museums have built tissue collections and made digital information (e.g., photographs, publications, geographic coordinates) freely available on the Internet. These collections provide endless opportunities to conduct studies, including temporal and spatial surveys of emerging and reemerging pathogens (*1*). We report an example of a museum collection being useful in detecting *Trypanosoma cruzi*, the etiologic agent of Chagas disease, in the southern plains woodrat (*Neotoma micropus*) in southern Texas. This finding is of interest in the epidemiology of Chagas disease because the climatic characteristics and demographics of the region are similar to areas in Latin America where Chagas disease is an important zoonotic agent that infects ≈20 million persons (*2*).

Tissue samples from *N. micropus* woodrats archived in the Natural Science Research Laboratory at the Museum of Texas Tech University were evaluated for *T. cruzi* DNA by PCR methods. All samples were originally collected during March 2001–June 2003 from the Chaparral Wildlife Management Area in southern Texas (28°18′N, 99°24′W), 86 km west of the Mexico–US border; some samples had been used previously in other research projects (*3*). Individual rodents were captured with live traps (n = 13) or by excavating middens in which all the nest occupants were collected by hand (n = 146). Animals were later euthanized and tissue samples (heart, kidney, liver, lung, muscle, spleen) were obtained. Tissues were immediately frozen in liquid nitrogen and permanently stored in ultralow-temperature freezers. We extracted 1 DNA sample from each animal's liver for use in this survey. DNA amplification was performed by using primers specific to *T. cruzi* (TCZ1 and TCZ2) (*4*) under previously standardized conditions and positive controls (*5*). *T. cruzi* DNA was detected in 42 (26.4%) of 159 woodrat samples tested. Males were infected significantly more often (31/82) than females (11/73); sex was not determined for 4 individuals (Score test for a binomial proportion, $z = -4.0$, $p<0.01$). Adults had a nonsignificant higher prevalence (24/92) than all other individuals in the remaining age categories combined (14/54) (age was not determined for 13 individuals) (Score test for a binomial proportion, $z = -0.02$, $p = 0.98$). Middens that harbored infected individuals (n = 28, mean = 1.8) were not significantly ($t = 0.79$, $df = 84$, $p = 0.43$) more populated than middens that harbored uninfected individuals (n = 58, mean = 1.6).

Woodrats had been shown by using microscopy to be infected by *T. cruzi* and *T. cruzi*–like organisms (*6*); however, no definitive DNA-based confirmation had been performed (*6*,*7*). The results of this research confirm the infection of *N. micropus* woodrats with *T. cruzi* and show a higher prevalence than that reported in previous studies that used other diagnostic methods. These results also point to woodrats as a potentially important reservoir of *T. cruzi* in North America. We hypothesize that the high prevalence is a consequence of the nest-building habits of these rodents. These nests are complexes of dry branches, grasses, and leaves, with a mean diameter of 84 cm, and offer easy access and permanent refuge to triatomine bugs. Woodrats have been found in association with at least 5 triatomine species: *Triatoma gerstaeckeri*, *T. lecticularia*, *T. neotomae*, *T. protracta*, and *T. sanguisuga* (*8*). Another factor for consideration is woodrats' multigenerational midden use, which may enable the permanent occurrence of triatomine colonies and therefore maintain long-term circulation of *T. cruzi*. Whereas recent characterizations of North American strains have included isolates from other mammalian reservoir hosts (*9*), the genotyping of parasites from *N. micropus* woodrats and other woodrats is still to be done.

Despite successful results from tracking pathogens by using material deposited in natural history museum collections (*10*), this practice is not common. We suggest that natural history museum collections be used more frequently, especially for surveying and genotyping *T. cruzi* in mammals, because of the importance of such information in clarifying the epidemiology and the evolutionary history of this pathogen.

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Aggression and Rabid Coyotes, Massachusetts, USA

To the Editor: In 1959, coyotes (*Canis latrans*) were found in only 3 Massachusetts towns, but by 2007, their population was estimated at 10,000 and they were present throughout the state, except on the islands of Martha's Vineyard and Nantucket (*1*). The coyote is highly adaptable and readily tolerates living near humans (*2*). Because the raccoon rabies virus (RRV) variant is endemic to Massachusetts and spillover into the coyote population occurs (*3*), coyotes are a potential source of rabies exposure for humans. Rabies in coyotes has emerged in Massachusetts at the same time that coyote and human populations have increased. From 1985 through 2008, the Massachusetts Department of Public Health tested coyotes by following the standard direct fluorescent antibody testing protocol published by the Centers for Disease Control and Prevention (*4*).

Of the 111 coyotes submitted for rabies testing, 4 (3.6%) were unsatisfactory because of decomposed brain tissue. Of the remaining 107 coyotes, 10 (9.0%) were found to be rabid; strain typing confirmed all 10 to have had spillover RRV. Within each county, the time between the first identification of RRV in an animal and finding a rabid coyote within that county ranged from 558 to 4,857 days; median was 2,799 days. The long time before spillover from raccoon to coyote was detected suggests that coyotes might avoid rabid reservoir animals. The time lag may also be the result of the distinct ecologic niches of these animals; coyotes are the top predators in ecosystems, and raccoons are only 1 of several mesocarnivores.

The public health rabies surveillance system in the United States is passive and relies on interaction of humans or domestic animals with rabies vector species (*5*). Because a rabid wild animal would go untested if a human or domestic animal had not had potentially infectious contact with it, the 10 coyotes with confirmed rabies likely represented only some portion of all rabid coyotes in Massachusetts during the study period.

Among 97 nonrabid coyotes, 7 had reportedly been in contact with humans and domestic animals. Among the 10 rabid coyotes, 4 were reported to have been in contact with humans and domestic animals. The coyotes in contact with both were 8.6× more likely to be rabid than were those in contact with only 1 or the other ($p<0.05$).

Of the 111 coyotes submitted for testing, the reported circumstances of potentially infectious contact were as follows: capture (n = 5), dead animal contact (n = 1), fight (n = 11), handling (n = 26), provoked attack (n = 1), specimen preparation (n = 3), unprovoked attack on a human (n = 4), vicinity (n = 5), unknown (n = 47), and other (n = 8). The proportion of coyotes with positive rabies test results varied by type of contact as follows: fight (5/11), handling (1/26), unprovoked attack (2/4), and unknown (2/47). Likelihood of being rabid was 15.2× ($p<0.0001$) and 11.9× ($p<0.05$) higher for coyotes reported with fight contact and unprovoked attack behavior, respectively, than for coyotes with any other reported contact. Biting as type of contact was reported for 18 coyotes; positive rabies test results were found for 7. Coyotes that had reportedly bitten a person or domestic animal were 18.2× more likely to be rabid than were coyotes that had not ($p<0.0001$).

Of 11 coyotes for which aggression was reported, 6 had positive rabies test results (Table); aggressive animals were 27.6× more likely to be rabid than were those not reported to be aggressive ($p<0.0001$). These findings provide statistical support for anecdotal reports (from as early as 1958) of rabid coyotes showing aggression (*6*). The following were significantly associated with a positive rabies test result for submitted coyotes: having had contact both with humans and with domestic or companion animals, having attacked a person without provocation, having fought with dogs, and having bitten either a person or domestic animal. This association between aggressive behavior and a positive rabies test result is of particular concern because of coyotes' relatively large size, their dramatically increased population, and their distribution throughout the state encompassing rural, suburban, and even urban areas. These factors increase the likelihood that a rabid animal will have the opportunity to interact with humans or their domestic animals, thus increasing the risk for rabies transmission.

A limitation of our study is the fact that the descriptions of the circumstances surrounding human interactions with a coyote were provided by members of the general public. Coyotes are large and unfamiliar animals, and such reports are likely to be distorted by that unfamiliarity and the fear engendered by the interaction. Another limitation is that the reported clinical signs represent only a proportion of coyotes that were submitted for testing, usually those that had had potentially infectious contact with a human or domestic animal.

Data involving coyotes from other states would be of interest because of the ongoing spread of RRV and the variation in coyote habitat and population. As populations of coyotes in many areas of dense human population increase, the risk for rabies and aggressive behavior in coyotes presents challenges for public health and animal management.

Table. Reported signs of disease in 111 coyotes submitted for rabies testing, Massachusetts, USA, 1985–2008*

Clinical sign	Total no. (%)	DFA result		
		Positive	Negative	Unsatisfactory
Aggression				
No	100 (90.1)	4	92	4
Yes	11 (9.9)	6	5	0
Ataxia				
No	109 (98.2)	10	95	4
Yes	2 (1.8)	0	2	0
Disorientation				
No	94 (84.7)	8	83	3
Yes	17 (15.3)	2	14	1
Found dead				
No	96 (86.5)	8	86	2
Yes	15 (13.5)	2	11	2
Lethargy				
No	96 (86.5)	10	83	3
Yes	15 (13.5)	0	14	1
Paralysis				
No	106 (95.5)	10	92	4
Yes	5 (4.5)	0	5	0
Salivation				
No	108 (97.3)	9	95	4
Yes	3 (2.7)	1	2	0
Seizures				
No	109 (98.2)	9	96	4
Yes	2 (1.8)	1	1	0
Wound of unknown origin				
No	98 (88.3)	10	84	4
Yes	13 (11.7)	0	13	0

*DFA, direct fluorescence antibody; unsatisfactory, not tested because of decomposed brain tissue; no, not observed or unknown.

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Neisseria meningitidis Serogroup X Sequence Type 2888, Italy

To the Editor: *Neisseria meningitidis* serogroup X was first described in the 1960s and has been found to be responsible of rare cases of invasive meningococcal diseases, in particular, meningitis, in North America, Europe, Australia, Africa, and the People's Republic of China (*1*–*3*). This serogroup has recently emerged in Africa as an increasing cause of meningitis; unfortunately, it is not covered by current vaccine programs. Serogroup X outbreaks have been reported in Niger, Ghana, and Kenya (*4*–*6*). In particular, in Niger during January–June 2006, *N. meningitidis* serogroup X represented 51% of confirmed cases of meningitis (*4*).

To investigate the population structure of serogroup X meningococci isolated during recent decades in Africa, Europe, and North America, Gagneux et al. (*1*) compared the molecular characteristics among them. That study highlighted a low genetic variability between African serogroup X strains, which contrasts with higher genetic variability among isolates from Europe and the United States (*1*).

We describe a case of invasive meningococcal disease caused by a serogroup X *N. meningitidis* strain isolated in Italy. The patient was a 55-year-old Italian woman with no immune deficiency. The onset of disease started quickly with high fever (39°C) on June 1, 2009. No contacts with persons coming from abroad were reported. This case was diagnosed on the basis of clinical signs and symptoms and results of laboratory confirmatory tests, including blood culture. The patient received ceftriaxone (2 g/day) for 7 days with a favorable outcome.

The strain was susceptible to penicillin G, rifampin, ciprofloxacin, and ceftriaxone, as determined by Etest method (bioMérieux, Florence, Italy). The breakpoints were those recommended by the Clinical and Laboratory Standards Institute (*7*). Serogroup was determined by serum agglutination, and serotype/subtype, NT:P1.15, 19 were determined by standard whole-cell ELISA with monoclonal antibodies (obtained from the National Institute for Biological Standards and Control, South Mimms, UK) (*8*).

PorA variable regions, FetA, and multilocus sequence typing analyses were performed according to standard procedures from the Neisseria Multi Locus Sequence Typing Web site (http://pubmlst.org/neisseria). The isolate from Italy had the pattern PorA VR1–19, VR2–15, and VR3–36; F5–5 and sequence type (ST)-2888. The same ST was already described in Greece in 2002 but in a noninvasive strain (http://pubmlst.org/neisseria).

The pattern obtained by pulsed-field gel electrophoresis (*9*), using the rare-cutting enzyme *Nhe*I, (data not shown), was identical to patterns found among meningococci X strains isolated in United Kingdom and belonging to ST-750, clonal group X-II (*1*). In particular, ST-2888 resembles, except for *gdh* gene sequence, ST-2317, which was found among the X meningococci isolated in the United Kingdom in 2002 with phenotype X:4:P1.7 (http://pubmlst.org/neisseria).

Our data document a rare case of invasive meningococcal meningitis in Italy, caused by *N. meningitidis* serogroup X ST-2888. Future surveillance data may be able to determine epidemiologic influences, likely emanating from nearby countries, on the spread of such a strain into Italy.

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Antiphospholipid Syndrome and Acute HIV Infection

To the Editor: Patients with acute HIV infection frequently experience a syndrome characterized by fever, sore throat, lymphadenopathy, maculopapular rash, and lymphomonocytosis, which mimics acute infectious mononucleosis, 3–6 weeks after primary infection (*1*). Aseptic meningitis, encephalitis, and peripheral neuropathy are the most commonly observed features. In contrast, antiphospholipid syndrome complicated with pulmonary emboli is not commonly associated with acute retroviral syndrome. The following case should prompt clinicians to consider an expanded clinical scope of initial signs and symptoms for acute HIV infection.

A 28-year-old homosexual man was admitted to a hospital in Madrid, Spain, on June 22, 2009, with fever, pharyingitis, and myalgias. Generalized lymphadenopathy was found on examination. Lymphomonocytosis and mild elevation of serum aspartate aminotransferase and serum alanine aminotransferase levels were found. Chest radiographs showed no abnormalities. Results of a commercial ELISA for HIV-1 and HIV-2 were negative. Results of a p24 antigen-capture assay were positive, and viral load measured by reverse transcription–PCR (RT-PCR, Amplicor; Roche Molecular Diagnostics, Pleasanton, CA, USA) was 2,600,000 copies RNA HIV/mL. CD4+ T-cell count was 297 cells/μL.

The patient was discharged with instructions to take acetaminophen, but he was readmitted 1 week later with recurring fever, pleuritic chest pain, and shortness of breath. He was febrile (38.5ºC), tachycardic, and tachypneic and had a blood pressure of 155/72 mm Hg and generalized lymphadenopathy. Blood tests showed a hemoglobin level of 10.6 g/dL, leukocyte count of 5,160 cells/μL, and thrombocyte count of 293 cells/μL. Results of renal function tests were within normal limits as were serum aminotransferase levels. Lactate dehydrogenase level was 698 IU/L (reference range 211–423 IU/L) and D-dimer was 3,414 μg/L (reference range 68–494 IU/L). Fibrinogen levels, prothrombin time, and partial thromboplastin time were normal. Chest radiographs showed a small area of pleural effusion on the left side. A computed tomographic scan of the chest showed multiple pulmonary emboli with areas of parenchymal infarction.

Antibodies against phospholipids (PLs) and β_2-glycoprotein I (β_2GPI) measured by ELISA were detected at high titers: immunoglobulin (Ig) M anticardiolipin + 72 U MPL/mL (positive at >20 U MPL/mL), IgG anticardiolipin + 158 U GLP/mL (positive at >20 U GLP/mL), IgG anti-β_2GPI + 210 U/mL (positive at >10). Results of screening tests for thrombophilia and other autoantibodies were within normal limits.

The patient was treated with low molecular weight heparin, oxygen, and analgesics. His fever subsided, and he was discharged a few days later while continuing to receive acenocoumarol, an oral coumarin anticoagulant. Results of a repeated HIV ELISA were then positive. Western blot assay confirmed the presence of antibodies to p24, gp41, and gp120/160.

One month after discharge, the patient was doing well. Titers of PL antibodies had declined (IgM anticardiolipin, negative; IgG anticardiolipin, 54; IgG antibody against β_2GPI 90). Viral load was 762,000 copies of HIV-1 RNA/mL, and CD4+ T-cell count was 320 cells/μL. At follow-up, 2 months after symptom onset, he was asymptomatic, and PL antibody titers continued to decline; antibodies against β_2GPI were undetectable, and only IgG anticardiolipin was still detected at lower titers (+33). Viral load was 129,000 copies/mL, and CD4+ lymphopenia was slowly improving (408 cells/μL). He was receiving anticoagulant therapy but not antiretroviral drugs.

Antibodies against PLs have been commonly found in patients with autoimmune diseases such as systemic lupus erythematosus and primary antiphospholipid syndrome, in which clinical manifestations (mainly thrombotic events) have been directly attributed to antibodies against PLs. In these patients, antibodies against PLs are specific for a neoepitope constituted by the union of β_2GPI, a lipid-binding coagulation inhibitor, to the cellular membrane phospholipids (*2*). In addition, these antibodies have been observed in some acute viral and bacterial infections as a manifestation of the intense antigenic stimulation of the immune system. These antibodies recognize lipid components of cellular membrane and have no direct role in the coagulation pathway, and their presence probably reflects intense antigenic stimulation of the immune system. Because of the lack of a statistical association between these antibodies and development of thrombotic events, the presence of these antibodies is thought to be an epiphenomenon and of no clinical relevance (*3*,*4*).

Anticardiolipin antibodies and, less frequently, β_2GPI antibodies also have been found in patients with chronic HIV infection, but their association with thrombotic events has not been proven (*5*). However, cases of antiphospholipid syndrome in HIV-infected patients have been anecdotally reported, prompting clinicians to reconsider the real role of these antibodies, particularly B_2GPI antibodies, which are thought to be more specific for antiphospholipid syndrome. Avascular bone and cutaneous necrosis and deep vein thrombosis and pulmonary emboli were the most common manifestations of antiphospholipid syndrome (*6*–*8*).

In HIV-infected patients, PL antibodies and β_2GPI antibodies have been strongly linked with level of viral replication (*9*). In our patient, the levels of viral load and PL antibodies seemed to run in parallel, with high concentrations of both at hospital admission and simultaneous decline over time. This observation suggests that patients with high levels of viremia, such as those with acute retroviral infection, could be at risk for high titers of PL antibodies and thrombotic events. Testing for antibodies in these patients should be considered as part of routine examination.

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Mycobacterium tuberculosis Beijing Strain, Bamako, Mali

To the Editor: *Mycobacterium tuberculosis* has ≥36 identified genotype families (*1*). Four genotypes cause 35% of documented cases of active tuberculosis (TB): Beijing (10%–11%), Latin American–Mediterranean (9.3%), Haarlem (7.5%), and the X clade (7%) (*1*,*2*) The Beijing clade strains, reported in 1995 from the People's Republic of China, are widely recognized as highly pathogenic with a possible predilection for multidrug resistance (*3*). Predominant in Asia, these strains have been documented in other parts of the world (*1*,*4*,*5*). The virulence, propensity to become resistant, and distinct geographic distribution of the Beijing clade suggest it may have some adaptive advantage in producing disease in humans. Limited data suggest that its presence in Africa is low (*2*,*4*,*5***)**.

In Bamako, Mali, 2 patients with active pulmonary TB came to the research clinic at Point G Hospital, affiliated with the University of Bamako Medical School, for recruitment under a US National Institute of Allergy and Infectious Diseases' institutional review board–approved protocol. The first patient, a previously healthy 34-year-old man, sought treatment in March 2008. He had a 3-month history of fever, cough, shortness of breath, and left-sided chest pain; respiratory rate of 24/min; temperature of 36.8ºC; and pulse rate of 68/min. He weighed 60 kg. His leukocyte count was 8,700 cells/μL, and he was positive for HIV-1 with a CD4+ T-cell count of 468 cells/μL. He reported contact with persons from other countries in Africa, China, and other parts of Asia.

Chest radiograph showed a cavitary lesion on the left upper lobe and opacities throughout the left lung. Three sputum samples collected 3 days apart were digested and decontaminated with N-acetyl-L-cysteine, 4% NaOH; concentrated by high-speed centrifugation; stained with auramine-rhodamine; and evaluated by using fluorescent microscopy. The many acid-fast bacilli (AFB) seen were identified by using nucleic acid probes (AccuProbe, Gen-Probe, San Diego CA, USA). Antimycobacterial drug susceptibility was determined by using a manual indirect susceptibility test (mycobacterial growth indicator tube, AST SIRE System; BBL, Becton Dickinson, Franklin Lakes, NJ, USA) showed the isolate sensitive to isoniazid (0.1 μg/mL), rifampin (1.0 μg/mL), and ethambutol (3.5 μg/mL) but resistant to streptomycin (0.8 μg/mL). Spoligotyping using a commercially available kit (Spoligotyping Isogen Life Science, De Meern, the Netherlands) showed characteristics of the Beijing clade (online Appendix Figure, panel A, www.cdc.gov/EID/content/16/2/362-appF.htm) (*6*).

The patient began treatment with the standard first-line regimen of isoniazid, rifampin, pyrazinamide, and ethambutol fixed-dose combination (Svizera Laboratory, Mumbai, India) according to Malian National Guidelines. Follow-up sputum samples at 13 and 18 weeks of treatment were smear- and culture-negative for AFB.

The second patient, a 28-year-old woman, sought treatment in July 2008. For 1 year, she had received first-line and retreatment regimens that failed to clear her sputum of AFB. She had begun second-line treatment for multidrug-resistant disease 2 days earlier. She had a history of fever, cough, and weight loss; temperature of 37.1ºC; heart rate of 104 beats/min; respiratory rate of 24/min; and blood pressure of 90/60 Hg mm. She weighed 49 kg. Leukocyte count was 9,400 cells/μL. Serologic results for HIV-1 and -2 were negative. Chest radiograph showed a right apical cavitary lesion and a fibrotic lesion in the right middle lung field. She did not recall any exposure to TB. She worked as an assistant at a local telephone center.

Two sputum samples, processed as described above, were positive for, and Gen-Probe testing confirmed, *M. tuberculosis*. According to antimycobacterial susceptibility testing, the strain was resistant to isoniazid (0.1 μg/mL), rifampin (1.0 μg/mL), ethambutol (3.5 μg/mL), and streptomycin (0.8 μg/mL). Spoligotyping confirmed the strain as Beijing clade, and restriction fragment length polymorphism (7) confirmed that it differed from that of patient 1 (online Appendix Figure, panel B).

The relevance of different genotypes, such as the Beijing clade, to disease progression is being studied. Evidence indicates the genotype may factor in transmission or pathogenesis. In a study in Cape Town, South Africa, disease produced by the Beijing clade increased exponentially over time, suggesting a possible pathogenic advantage; although most cases were drug susceptible, the likelihood of unsuccessful treatment was greater than for non-Beijing variants (*8*). Although the Beijing clade does not appear to have greater propensity than non-Beijing genotypes for acquiring resistance, certain variants within the group that become multidrug resistant may be more likely to acquire such resistance. Beijing strains particularly may tend to acquire resistance more easily than others under conditions of suboptimal treatment (*9*). In Cape Town during 2000–2003, the Beijing clade as a cause of disease in children increased from 13% to 33%, suggesting a selective advantage in transmissibility and disease production (*10*).

These cases highlight the need to diagnose disease and resistance early and to begin appropriate treatment in TB-endemic countries. Knowledge of circulating strains and their resistance patterns is essential to developing

effective programs to curtail the spread of TB within the country and the region; in this era of globalization, it is required for the successful control of TB worldwide.

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Hemorrhagic Fever with Renal Syndrome, Vietnam

To the Editor: Hantaviruses are primarily rodent borne and can cause hemorrhagic fever with renal syndrome (HFRS) in persons who inhale aerosolized excreta from infected rodents. The clinical characteristics of HFRS are fever, hemorrhage, and varying degrees of renal and hepatic dysfunction. Although HFRS is endemic primarily to Eurasian regions, there is serologic evidence of hantavirus infections in rodents and humans worldwide (*1*). Little is known about the occurrence of hantavirus infection in rodents or humans in Vietnam. One study found 5.4% prevalence of antibodies against Hantaan 76–118 and Puumala strains among residents of the Hanoi Metropolitan (*2*), whereas another study in southern Vietnam did not find evidence of hantavirus infection in humans (*3*). We describe autochthonous HFRS from Vietnam, possible reservoir hosts, and the follow-up investigation, which implies the presence of a strain of Seoul virus (SEOV).

The case-patient was a previously healthy 25-year-old nurse working in a referral hospital and residing in a semiurban district of Ho Chi Minh City. On September 23, 2008, she was admitted to the referral hospital with a history of high fever, chills, myalgia, nausea, vomiting, hematuria, and abdominal and lower back pain for 3 days. Physical examination showed a body

temperature of >39°C, petechiae, mild dehydration and hypotension, with otherwise unremarkable vital signs. Hematologic tests showed 13,300 leukocytes/mm^3, 167,000 thrombocytes/mm^3, and hematocrit of 31%. Urinalysis showed grave hematuria (3+), proteinuria (2+), and leukouria (2+).

Three days after admission, acute renal failure with relative oliguria (0.85 L/24 h) developed, as well as uremia (26.4 mg/dL), creatinemia (0.98 mg/dL), and abnormal liver function (aspartate aminotransferase 49 U/L and alanine transferase 60 U/L). The following day the patient had dyspnea and became agitated. Ultrasound examination showed pleural effusion, parietal pericardial effusion, peritoneal ascites, hepatomegaly, and renal thickness. Six days after admission, diuretic problems developed in the patient (3.7 L/24 h), her dyspnea resolved, and she became afebrile. Ten days after admission, the patient's hematuria resolved, and renal and liver functions gradually recovered; she was discharged after 29 days of hospitalization.

Immunoglobulin (Ig) M and IgG against Hantaan recombinant nucleocapsid protein antigen were detected in the case-patient's acute-phase and convalescent-phase serum samples, respectively, by ELISA (*4*,*5*). The presence of antihantavirus IgG was confirmed by immunofluorescent antibody (IFA) assay using whole hantavirus antigen and Western blot using hantavirus CL-1 strain (*6*,*7*). In further analysis, neutralization antibodies against SEOV strain SR-11 were detected by focus reduction neutralization test (*8*). The viral RNA, however, was not detectable in the acute-phase blood sample by reverse transcription–PCR (RT-PCR) (*9*). Other serologic tests were performed for dengue fever, typhoid fever, hepatitis B, and malaria; results of culture of blood and urine were negative for bacteria.

Following confirmation of the diagnosis, close contacts of the patient were investigated. Two family members of the patient did not have any symptoms compatible with HFRS; their serum samples were tested and found negative for antihantavirus IgG. Because the patient was a nurse, possible nosocomial transmission and sources were also investigated and excluded. On the basis of the patient's strong history of exposure to rodents at home, further investigation focused on the domestic rodent population.

From October 14 through 16, 2008, 110 rodent traps were set within and surrounding the patient's house. The total catch was 32 rodents, of which 16 were *Rattus norvegicus*, 7 *R. exulans*, 5 *R. argentiventer,* and 4 *Bandicota indica*, respectively. By using ELISA, IFA, and Western blot, antihantavirus IgG was detected in serum from 7 rats, of which 5 were *R. norvegicus*, 1 *R. argentiventer*, and 1 *B. indica*. Further analysis using RT-PCR identified 2 SEOV strains from *R. norvegicus and R. argentiventer* captured in the patient's house. The M segment of 1 identified SEOV strain (24D1208) was sequenced and compared with 22

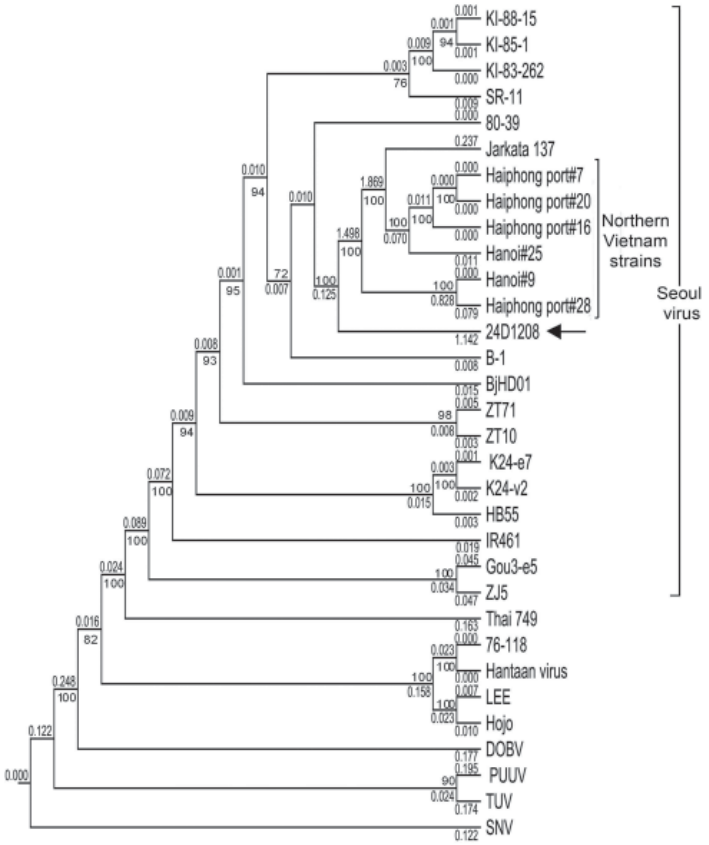

Figure. Phylogenetic tree (CLC-Combined Workbench 3) showing partial sequences of the medium segment (nt 810–2355). The newly identified Seoul virus (SEOV) was denoted as 24D1208 (arrow). The M segment sequences of the reference strains are: SEOV strains KI-88-15 (D17594), KI-85-1 (D17593), KI-83-262 (D17592), SR11 (M34882), 80–39 (S47716), Jakarta137 (AJ620583), Haiphong port #7 (AB355728), Haiphong port #20 (AB355730), Haiphong port #16 (AB355729), Hanoi #25 (AB355733), Hanoi #9 (AB355732), Haiphong port #28 (AB355731), B-1 (X53861), BjHD01 (DQ133505), ZT71 (EF117248), ZT10 (DQ159911), K24-e7 (AF288652), K24-v2 (AF288654), HB55 (AF035832), IR461 (AF458104), Gou3-e5 (AF288650), and ZJ5 (FJ811839); Thailand virus strain 749 (L08756); Hantaan virus strains 76–118 (M14627), Hantaan (NC005219), LEE (D00377) and Hojo (D00376); Dobrava virus (DOBV) strain Dobrava (L33685); Puumala virus strain Sotkamo (X61034); Tula virus (TUV) strain Tula/Moravia/5302v/95 (Z69993); and Sin Nombre virus (SNV) strain NMH10. The numbers at the nodes are bootstrap confidence levels for 1,000 replications. Only bootstrap support values >70% are shown.

SEOV strains, 6 of which were from *R. norvegicus* rodents captured in urban areas of North Vietnam. Phylogenetic analysis showed that this SEOV belonged to the Vietnamese SEOV genotype (Figure).

We describe a clinical case of hantavirus infection and its potential rodent reservoir occurring in Vietnam. The clinical manifestations of the case-patient were compatible with SEOV infection, which is responsible for a moderate form of HFRS (*10*). Also, HFRS caused by SEOV occurs in urban rather than rural areas, unlike other hantavirus infections. Our epidemiologic findings were compatible with other studies indicating the source of infection was the case-patient's home, the only place where she had a history of exposure to rodents. Although viral RNA could not be obtained from the case-patient for genotyping, the genomic comparison of the viral strains from rodents captured in the case-patient's home and elsewhere in Vietnam suggested that the source of infection was local rodents. This report provides additional evidence that hantavirus infection is a worldwide problem and is likely underdiagnosed in Vietnam and other countries where simple standardized laboratory diagnostics are not widely available.

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Origin of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus, China

To the Editor: A highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV), which affected >2 million pigs, emerged in early 2006 in the People's Republic of China. The disease was characterized by high fever (41°C), high illness rates (50%–100%), and high death rates (20%–100%) for pigs of all ages (*1*). A number of HP-PRRSVs have been isolated from 2006 through 2009 from infected pigs in different provinces of China and confirmed to be the causative agent of the new outbreaks (*1*,*2*). These HP-PRRSVs have a deletion of 30 amino acids in nonstructural protein 2 (NSP-2). However, the evolutionary origin and path of the HP-PRRSV remain unknown.

We analyzed the full-length sequences of 67 PRRSVs: 35 HP-PRRSVs (HuN4 and LNSY-08-1 isolated in our laboratory and 33 viruses isolated in other laboratories), 28 classic PRRSVs (18 viruses isolated from China and 10 viruses representing other Asian countries and North

America), and 4 commercially available attenuated live PRRSV vaccine viruses. Except for the 2 viruses we isolated (HuN4 and LNSY-08-1), the full-length sequences of the other 65 viruses were obtained from GenBank. Nucleotide and deduced amino acid sequences of these PRRSVs were aligned and compared by using previous methods (*3*,*4*).

Whole genome–based phylogenetic analysis showed that these 67 PRRSVs could be divided into 4 subgroups (online Appendix Figure, www.cdc.gov/EID/content/16/2/365-appF.htm). Ten classic PRRSVs from China, together with the North American prototype virus VR-2332 and the vaccine virus RespPRRS/Repro modified live vaccine, were classified into subgroup 1. The first Chinese isolate, CH-1a, and its 3 derivatives (CH2002, CH2003, and CH2004) were classified into subgroup 2. All 35 HP-PRRSVs were classified into subgroup 4, and they shared high homology (>99%) in their genomic sequences. The other 4 Chinese PRRSVs, including HB-1(sh)/2002, HB-2(sh)/2002, Em2007, and SHB, belonged to subgroup 3, an intermediate subgroup between subgroups 2 and 4. Phylogenetically, HP-PRRSVs had a close relationship with subgroups 2 and 3.

Four conserved deletions were shown among all HP-PRRSVs, including an adenosine deletion at position 122 in the 5′-untranslated region, a guanosine deletion at position 15,278 in the 3′-untranslated region, and 2 discontinuous deletions in the NSP-2, including a single amino acid deletion at position 482 (L^{482}) and a second deletion of 29 amino acids between positions 533 and 561 (S^{533}–A^{561}). The presence of these 4 deletions among subgroup 4 viruses is a unique phenomenon, which may be used as a distinctive molecular marker for HP-PRRSVs.

The occurrence of these 4 deletions might be explained as a stepwise accumulation from subgroup 2 to subgroup 4. None of the 4 deletions were found in subgroup 2. Among viruses in subgroup 3, one, 2, or 3 of the 4 deletions occurred. For example, a single deletion was present at 122 nt in Em2007, double deletions at 122 nt and 15,278 nt in HB-1(sh)/2002 and SHB, and triple deletions at 122 nt, 15,278 nt, and 482 aa in GD3-2005 (this sequence was not submitted to GenBank until now). In 2008, Ma et al. compared GD3-2005 with several PRRSVs and reported the homology within them, pointing out that the 2 deletions in NSP-2 were identical to the HP-PRRSV (*5*). After careful analysis, we found the GD3-2005 more interesting than what was reported by Ma et al.; it belongs to an intermediate group, and shares the characteristics of gradual evolution. Eventually, all 4 deletions occurred in subgroup 4. This obvious pattern suggests that these 4 conserved deletions might have evolved step by step.

The primary neutralizing epitope (PNE), which is located on glycoprotein 5 and composed of the residues S^{37}H(F/L)QLIYN with F/L^{39} as the binding site for the neutralizing antibody (*6*,*7*), also displayed similar changes at the 39 position among the 4 subgroups. The PNE residues in subgroups 1 (SHL^{39}QLIYN) and 2 (SHF^{39}QLIYN) were considerably conservative. Subgroup 3 contained either F^{39} or I^{39} (F^{39} in Em2007 and HB-2(sh)/2002, and I^{39} in both HB-1-(sh)/2002 and SHB); subgroup 4 contained I^{39} only. The existence of either F^{39} or I^{39} in subgroup 3 PNE indicates its intermediate position between subgroups 2 and 4 in the evolution of HP-PRRSVs.

Pairwise comparison of subgroups 2, 3, and 4 did not find recombination or large fragment replacement, which suggests that all HP-PRRSVs originated from the same ancestor by gradual evolution. Notably, the recently isolated intermediate PRRSVs mentioned above (SHB, Em2007, and GD3-2005) were isolated in the region of South China where the outbreak of HP-PRRS initially occurred. Furthermore, the epidemiologic data show that the outbreak of HP-PRRSV emerged from 1 particular place and then spread widely. This evidence indicates that all HP-PRRSVs isolated in China likely originated from the same source.

In summary, our findings suggest that the newly emerged HP-PRRSVs originated from the Chinese CH-1a-like PRRSV. Further study is needed to determine what contributes to the increased pathogenicity of HP-PRRSV. Although the 4 deletions are conserved in all HP-PRRVs, the increased pathogenicity of HP-PRRSV may not merely be caused by the deletions; pathogenicity is affected by multigenetic factors.

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Evidence-based Tool for Triggering School Closures during Influenza Outbreaks

To the Editor: I read with interest the recent article by Sasaki et al., "Evidence-based Tool for Triggering School Closures during Influenza Outbreaks, Japan" (*1*), which describes an algorithm for determining the optimal timing of school closures to control influenza outbreaks. The published information is a helpful guide for predicting influenza outbreaks in school settings. However, no data are presented to show the efficacy of school closures after the detection of such outbreaks. As such, the title "Evidence-based Tool for Predicting Influenza Outbreaks, Japan" would more accurately describe the article.

The findings presented by Sasaki et al. (*1*) could be used to help make a decision for school closure or dismissal in places like Japan, but no information is provided on whether this approach is effective in preventing further influenza virus transmission. This is an important distinction and should not change the current school response guidance published by the Centers for Disease Control and Prevention (CDC) (*2*). In general, CDC guidance suggests that during an influenza outbreak, policymakers should weigh the advantages and disadvantages of school dismissals or school closures before making a decision.

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In Response: Vogt (*1*) correctly points out that our article (*2*) did not present data on the effectiveness of school closures to control influenza outbreaks. However, public health agencies continue to support school closure as a nonpharmaceutical response to the ongoing outbreak of pandemic (H1N1) 2009 (*3*) despite little evidence for the appropriate timing of closures, even though it is known that timely action is critical. As the title of our article reflects, our algorithm was designed as an evidence-based tool for supporting the timing of school closures.

In our article, we pointed out that evaluating the impact of school closures is a critical research question. Before April 2009, decision-making regarding school closure in Japan was left to individual schools, 98% of which are public. Since then, recommendations for public school closure have been made according to standardized rules set by the Japanese School Health and Safety Law, leaving final decision-making authority up to local education boards. Our next study will evaluate the effectiveness of this early, standardized timing of school closure in Japan.

On September 24, 2009, the Japanese Ministry of Health, Labor and Welfare presented a school closure plan for use in the different stages of an influenza outbreak; the plan is based on World Health Organization

recommendations (*3*). In the early periods of an outbreak, when infection is not widespread, a "proactive school closure plan" will be put into effect; school closure for 5–7 days will be implemented as soon as the first infection is detected in a school. If the outbreak is already spreading through the community, "reactive school closure" is considered sufficient. This closure has been performed during seasonal influenza outbreaks in Japan, closing classes when many students were absent from school. Most schools now close when >10% infection-related absenteeism is reached. Our study, which provides a refinement of thresholds predictive of serious outbreaks in schools, may support this plan in Japan and provide schools worldwide with an approach to considering the timing of school closures.

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Personal Log, Stardate 42552.6

Curi Kim

Awaiting the alien delegation from Sarona VIII
To scan them for communicable space disease,
I think of history and can't help but now speculate
If our ancestral officers in duty were at ease
At their own quarantining stations, earthbound ports of entry,
There watchful for all ancient scourges crossing borders
(tuberculosis … measles … influenza … coxsackie),
Assessing travelers sans medical tricorders,
But using only judgment and with epi skills equip'd
To help determine meaning in a cough, a rash;
And knowing what they saw was barely the berg's tip
Considering all the infected travelers they didn't "catch"—
Ah, the delegates just beamed aboard to be met;
Let's see what interstellar parasites we detect!

Dr Kim is a quarantine medical officer at the CDC Detroit Quarantine Station, Detroit, Michigan. Her primary research interest is traveler's health.

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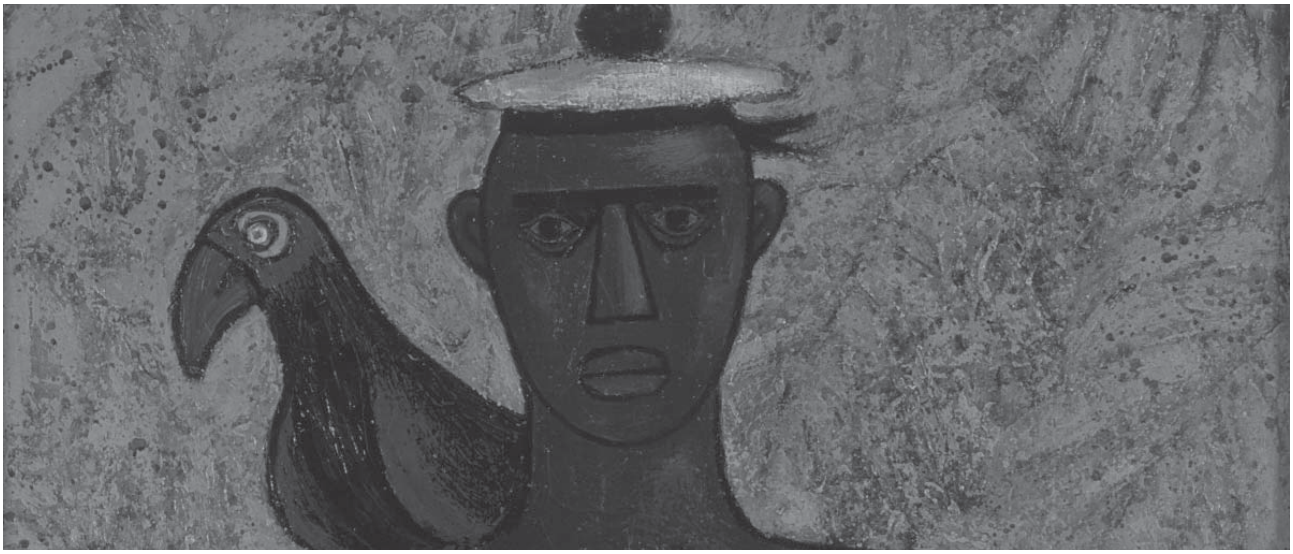

Ellis Wilson (1899–1977). Caribbean Bird Vendor (1953) (detail). Oil on canvas (91.44 cm × 60.96 cm). Hampton University Museum, Hampton Virginia

Bird's Eye View of Emerging Zoonoses

Polyxeni Potter

"So much to paint and so little time" was Ellis Wilson's assessment of his chosen profession. "I want to paint all the time—everything of interest and beauty." His approach was to recreate life around him, people at work. He painted them making turpentine, mining clay, harvesting tobacco, buying and selling goods. His portrait of an aircraft-engine factory worker in New Jersey won him a Guggenheim Fellowship in 1944. But his best known painting is probably Funeral Procession, a work displayed above the mantle of the Huxtables' living room in the 1980s–90s television series The Cosby Show.

Wilson was born in Mayfield, a cloth-manufacturing town in Kentucky's tobacco-growing region. The son of a barber and cabinet maker who took art lessons and painted with some success, he was inspired by his father to study art from a young age. "I guess I got what little talent I have from him," he said in a 1975 interview. Young Wilson did odd jobs and worked as janitor at Day's Ready-to-Wear, where his weekly portraits drawn with cleaning wax on the dress shop window delighted passersby.

The aspiring artist attended Kentucky State College in Frankfort, then moved north to continue his studies at the Art Institute of Chicago in 1919, a period of racial upheaval and riots. "I couldn't go downtown to the Art Institute. They were shooting and carrying on." He joined the Chicago Art League and met many in the art community, among them sculptor Richmond Barthé. "I had never been in a group of artists …. It was just great to be numbered among them." After completing his studies, he worked as commercial artist for a few years, then traveled to New York in 1928, during the period of cultural and artistic resurgence Harlem Renaissance.

In New York, Wilson broke away from the academic style taught him in Chicago and from representational painting to focus on shape and color. His forms became increasingly angular and elongated, the colors brighter and more vibrant, the faces spare and masklike with a modernist inflection. "I just cut out completely from anything that looked like a portrait. It was freer. I was astounded." He worked for the Federal Art Project sponsored by the Works Progress

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Administration and at various other jobs, joined the Harlem Artists Guild, and continued to paint and meet other painters: Aaron Douglas, Alain Locke, Joseph Delaney, Palmer Hayden. He moved to Greenwich Village, where he exhibited at Augusta Savage's Salon and other venues.

"Practically all my life I have been painting under difficult conditions," Wilson said of earning a living from art. "Lack of money and time, especially time, has prevented me from painting as much and as often as I have wanted to." Dealers "wanted all black painters to be 'primitives,'" and the public viewed him with mistrust. "A white woman who bought pictures from me invited me to her home in North Carolina. I told inquisitive people that I did painting for her in New York. They thought I was a house painter."

Despite these difficulties, Wilson sought and received fellowships, awards, and other opportunities, which allowed him to roam the southern states, the Sea Islands, and later Haiti. The experiences broadened his artistic scope and nourished his creativity. "I could express myself to the fullest degree and accomplish worthwhile work." In Charleston, South Carolina, he became fascinated with the local open market, its vendors and crowds and the hustle and bustle of their daily activities. He observed and sketched them freely and marveled at the authenticity and honesty of their lives. "I noticed such great hopes among the people in the South: hopes that they could soon vote, and hopes that education would become free and open. My own hope is that I capture their hopes in my work."

His work in Haiti, marked by exuberant color and figures free of extraneous detail, was similarly expansive, "I'd never seen a tropical place—and with the music, the drumming, the dancing… [the people] were very artistic." This work was well received back in New York, where he returned to live and paint until his death. But his legacy as an innovator, both in his choice of subject matter and geometric boldly colored form, was not fully appreciated until later.

"I hold my honey and I store my bread / In little jars and cabinets of my will / I label clearly, and each latch and lid / I bid, Be firm till I return from hell," wrote Gendolyn Brooks (1917–2000) in her poem "My Dreams, My Works, Must Wait Till after Hell," expressing the frustrations of her generation and giving words to Wilson's dilemma. All his training, high profile works, contacts in the art world, all his talent and dedication could not overcome the odds against him. He died unknown and was buried in an unmarked, "pauper's" grave. Fewer than 100 of his paintings have been located.

Caribbean Bird Vendor, on this month's cover, captures Wilson's love of beauty as a main goal in art. The flat fully delineated form suits the regal figure he has placed against yellowing vegetation and directly in front of the viewer. Sturdy and solid, this vendor is sketched with little embellishment or detail, yet his geometric features denote sadness, and he seems preoccupied. His eyes, unfocused, show detachment from his circumstances as a seller of birds. The parrot on his shoulder is perched with ease, claws withdrawn. The bird in the lower left is also at peace, nesting attentively in his cupped palm. But the one in the center is panic stricken. Pulling in the opposite direction from the other birds, its feathers ruffled and spread, beak open, eye glaring, claws flexing, it is ready for flight. The vendor seems ambivalent as he steadies it on his chest.

The vibrant colors only add to the tension in this scene. The bird in distress, the most attractive and volatile, draws all the attention, its frantic state amplifying the unnatural calm of the vendor, who seems not controlling but protective, as if uncomfortable with the intent to sell his charges. A means to earn a living, the sale of birds and other wildlife out of their natural habitat is part of economic reality in much of the world. This vendor may well loathe his own occupation.

Apart from the cruelty inherent in animal displacement and captivity, sensed by Wilson's vendor, other reasons, not least of them public health, compel against the sale and import of exotic birds. Unsanitary conditions in live bird markets in the United States and abroad have come under scrutiny in recent years, when legal and illegal trade in domestic and wild birds was associated with the global spread of highly pathogenic avian influenza (H5N1). This issue of Emerging Infectious Diseases features other health crises associated with animals on the move, from leptospirosis to Marburg virus infection and white-nose syndrome in bats, and examines fungal infections that could spread through inhaling dust contaminated with spores from bird droppings.

As he painted other people at work, Wilson was sensitive to the socioeconomic factors that prevented him from achieving greatness in his own time. What he did not know was that the same factors interfere with health, in a web of zoonotic and environmental interconnections.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Preparing a Community Hospital to Reduce Risk for Work-related Exposures to Exotic Agents

Bartonella spp. Transmission and Ticks

Potential for Tick-borne Bartonelloses

Vaccine Preventability of Meningococcal Clone, Europe

Chikungunya Virus Infection during Pregnancy, Réunion, France, 2006

Mumps Outbreak in a Hospital

New *Legionella pneumophila* Serogroup 1 Clones, Ontario, Canada

Invasive *Haemophilus influenzae* Disease, Europe, 1996–2006

Avian Bornavirus and Fulfillment of Koch's Postulates

Bloodmeal Analysis to Identify Reservoir Hosts for *Amblyomma americanum* Ticks

Serologic Markers for Detecting Malaria in Areas of Low Endemicity, Somalia, 2008

Infection of Kissing Bugs with *Trypanosoma cruzi*, Tucson, Arizona, USA

Borreliae, Ehrlichiae, and Rickettsiae in Ticks, Texas, USA

Murine Typhus in Austin, Texas, USA, 2008

West Nile Virus in American White Pelicans, Montana, USA, 2006–2007

Cost of West Nile Virus Outbreak, Sacramento County, California, USA, 2005

Pandemic (H1N1) 2009 Virus Infection in Domestic Cat

Complete list of articles in the March issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 19–21, 2010
2nd International Berlin Bat Meeting: Bat Biology and Infectious Diseases
Berlin, Germany
http://www.izw-berlin.de

March 18–22, 2010
Fifth Decennial: International Conference on Healthcare-Associated Infections 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.decennial2010.com

March 24–26, 2010
16th ISHEID (International Symposium on HIV & Emerging Infectious Diseases)
Marseille, France
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July 11–14, 2010
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Hyatt Regency Atlanta
Atlanta, GA, USA
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Article Title

Risk Factors for and Estimated Incidence of Community-associated *Clostridium difficile* Infection, North Carolina, USA

CME Questions

1. What was the approximate percentage of all *Clostridium difficile* infections (CDIs) that were community associated in the current study?

A. 2%
B. 7%
C. 20%
D. 57%

2. Which of the following demographic trends in community-associated (CA) CDI was present in the current study?

A. Adults over the age of 64 years had the highest prevalence of CA-CDI
B. Adults younger than 44 years had the highest prevalence of CA-CDI
C. Physician visits had no impact on the prevalence of CA-CDI
D. Women had a higher prevalence of CA-CDI compared with men

3. Which of the following case characteristics of CDI in the current study is most accurate?

A. Less than 10% of patients with CDI were admitted to the hospital
B. Diarrhea was the most common complaint
C. Nearly all patients had been exposed to antimicrobials in the 3 months prior to testing for CDI
D. Clindamycin was the antibiotic most frequently implicated in promoting CDI

4. All of the following variables were associated with a higher risk for CA-CDI in the current study, except:

A. Recent treatment with antimicrobial drugs
B. More outpatient visits at the VA hospital
C. Treatment with proton-pump inhibitors
D. Cardiac failure

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.